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## NUTRIENT MEDIATED PROTECTION AGAINST ENDOTHELIAL CELL DYSFUNCTION

Gudrun Reiterer

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ABSTRACT OF DISSERTATION

Gudrun Reiterer

The Graduate School

University of Kentucky

2004

NUTRIENT MEDIATED PROTECTION AGAINST  
ENDOTHELIAL CELL DYSFUNCTION

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ABSTRACT OF DISSERTATION

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A dissertation submitted in partial fulfillment of the  
requirements for the degree of Doctor of Philosophy at the  
Graduate Center for Nutritional Sciences  
at the University of Kentucky

By  
Gudrun Reiterer

Lexington, Kentucky

Advisor: Dr. Bernhard Hennig, Professor of Nutrition and Toxicology

Lexington, Kentucky

2004

## ABSTRACT OF DISSERTATION

### NUTRIENT MEDIATED PROTECTION AGAINST ENDOTHELIAL CELL DYSFUNCTION

Atherosclerosis is thought to be initiated by endothelial cell dysfunction. Research described in this dissertation is focused on interactions of nutrients, cytokines and pharmaceutical compounds in the intracellular signaling pathways leading to endothelial cell activation. The flavonoid quercetin could significantly downregulate the inflammatory pathways induced by linoleic acid as determined by DNA binding assays of the proinflammatory transcription factors nuclear factor-kappaB and activator protein-1 as well as by gene expression studies of interleukin-6 and vascular adhesion molecule-1. Interestingly, quercetin and vitamin E also prevented the linoleic acid-induced activation of PPAR DNA binding - suggesting a role of oxidation in the fatty acid-mediated induction of PPAR. In addition, we studied an interaction of zinc with the anti-inflammatory transcription factors, peroxisome proliferator activated receptors (PPARs) alpha and gamma. Our data suggest that PPAR alpha and gamma and their synthetic agonists require zinc for their antiinflammatory properties in endothelial cells. We could confirm the importance of zinc in PPAR gamma signaling *in vivo* by a decreased PPAR DNA binding activity in livers of zinc deficient mice. Furthermore, zinc had dramatic lipid lowering effects in LDL-receptor deficient mice on a diet rich in corn oil. Triglycerides, phospholipids and cholesterol levels were significantly elevated in mice receiving a zinc deficient diet when compared to control and were decreased in zinc supplemented animals. Zinc deficiency also increased oxidative stress as determined by

quantitation of plasma isoprostanes and mRNA expression of glutathione reductase. In conclusion, our data show novel interactions of proinflammatory nutrients, such as linoleic acid, with antioxidant and anti-inflammatory nutrients, such as quercetin and zinc.

**KEYWORDS:** Atherosclerosis, Zinc, Quercetin, PPAR, LDL-R<sup>-/-</sup> mice

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NUTRIENT MEDIATED PROTECTION AGAINST  
ENDOTHELIAL CELL DYSFUNCTION

By

Gudrun Reiterer

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Director of Dissertation

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Director of Graduate Studies

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DISSERTATION

Gudrun Reiterer

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NUTRIENT MEDIATED PROTECTION AGAINST  
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## Acknowledgments

I would like to thank the key persons who guided me throughout my career as a graduate student at the University of Kentucky. Most of all, I thank my advisor, Dr. Bernhard Hennig, who provided me with an environment that was productive and supportive for completing and enjoying my training as a Ph.D. student. He not only gave advice in research and academic matters but was also understanding and supportive in other aspects of life.

I would also like to express my gratitude to all the members of my Ph.D. committee; Drs. Alan Daugherty, Michael W. Kilgore and Michal Toborek who were very generous in sharing their expertise and support to any research related question I had during my time as a graduate student.

In addition, I would like to thank those individuals that contributed to the research projects I have been working on. Most of them are represented as coauthors in my research publications: Drs. Ruth MacDonald, Jim Browning, Deborah Rateri, Sergey Matveev, Jason Morrow, Eric Smart, as well as all my laboratory coworkers.

Last but not least I would like to acknowledge the support of my family, in particular my parents Guenter and Herta as well as Michael L. Hayes. Despite the long distance they played an important and very valuable part in my time at the University of Kentucky. Therefore, this work is dedicated to Michael and my parents.

The research reported in this dissertation has been funded by the American Heart Association predoctoral fellowship (0215075B) and by grants from NIEHS/NIH (ES 07380), USDA/NRI (2001-01054 and 00-35200-9101), National Cattlemen's Beef Association, Kentucky Cattlemen's Association and the Kentucky Agricultural Experimental Station.

# **NUTRIENT MEDIATED PROTECTION AGAINST ENDOTHELIAL CELL DYSFUNCTION**

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## Introduction

Since 1900 cardiovascular diseases have been the leading cause of death in the United States every year but 1918. Cardiovascular diseases are not only a problem in industrialized countries but are an upcoming problem in developing countries as well. The main risk factors are diabetes, hyperlipidemia, hypertension, excess body weight, physical inactivity and tobacco smoke.

Endothelial cells play a critical role in the initiation of atherosclerotic events. Endothelial cells form the innermost layer of blood vessels and are therefore in immediate contact with the blood and substances carried in the blood. Atherosclerotic events can be initiated by endothelial cell activation upon exposure to stimuli such as cytokines, oxidized or glycosylated macromolecules and blood pressure regulating substances (Ross 1999). The endothelium responds with an increased expression of adhesion molecules, interleukins and monocyte attracting molecules - thus promoting the diapedesis of monocytes into the subendothelial space and their differentiation into macrophages. Lipid-laden macrophages, known as foam cells, are major components of fatty streaks, the first visible manifestation of atherosclerosis (Ross 1999).

Nutrition helps prevent heart disease and stroke. Particularly lipids have a major role in the pathogenesis of atherosclerosis. These include not only oxidized lipoproteins, particularly oxidized LDL (low density lipoprotein), but also free fatty acids. The lipoprotein lipase on the luminal side of endothelial cells cleaves triglycerides carried in LDLs and VLDLs (very low density lipoproteins), thus exposing the endothelium to a high concentration of free fatty acids (Zilversmit 1979). Concentration levels ranging from 180 – 2,500  $\mu\text{mol/L}$  have been reported (Fredrickson and Gordon 1958). Certain polyunsaturated fatty acids, such as linoleic acid are prone to oxidation and can trigger oxidative stress sensitive inflammatory pathways in endothelial cells (Kok et al. 1991). Most of these inflammatory pathways result in the activation of the transcription factors NF- $\kappa$ B (nuclear factor- $\kappa$ B) and AP-1 (activator protein-1) with subsequent expression of cytokines and adhesion molecules (Hennig et al. 2001).

Prevention of cardiovascular diseases by nutrition and pharmacology frequently aims to inhibit the activation of these inflammatory pathways. For example, the anti-

inflammatory transcription factors PPAR $\alpha$  and  $\gamma$  (peroxisome proliferator activated receptors) are known to negatively interfere with the NF- $\kappa$ B, AP-1 and STAT (signal transducer and activator of transcription) pathways and can be targeted by pharmaceuticals to downregulate inflammatory pathways (Zhou and Waxman 1999, Delerive et al. 1999).

The prevention of the development of atherosclerosis by the means of nutrition does not usually target one specific pathway. Many bioactive molecules found in foods have several properties, some of them still unknown. For example quercetin, a flavonoid found in fruits and vegetables has antioxidant properties as well as kinase inhibitory properties (Metodiewa et al. 2001, Peet and Li 1999, Yoshizumi et al. 2002). It is also likely to interact with nuclear receptors such as the estrogen receptor or PPARs (Virgili et al. 2004, Reiterer et al. 2004a, Thuillier et al. 2002). Although their concentration in the bloodstream following food consumption is rather low – levels range in the high nmol/L to low  $\mu$ mol/L (Hollman and Katan 1997), these bioactive food compounds seem to be important contributors to the overall antioxidant and antiinflammatory defense systems in the cell.

In addition, nutrients can also be essential cofactors to enzymes involved in this antioxidant defense system. Zinc for example, is essential for the structure and function of a large number of macromolecules and for over 300 enzymatic reactions, including the Cu,Zn-SOD (superoxide dismutase) (Sarstead 1995). The role of zinc as an antioxidant has been well established. Zinc stabilizes thiol groups of proteins, thus protecting these sites from oxidative modification (Klotz et al. 2003). In addition to the largely fixed pool of zinc as a structural compound, zinc has also been suggested to act as a free signaling molecule - transiently interacting with cellular signaling pathways (Truong-Tran et al. 2000). While the role of this free zinc pool is not well defined, it is much more susceptible to alimentary zinc deficiency and supplementation.

The chapters of this dissertation address the interaction of various nutrients, such as linoleic acid, quercetin, zinc and vitamin E with intracellular pro-inflammatory signaling pathways, resulting in NF- $\kappa$ B and AP-1 activation as well as with anti-inflammatory pathways mediated by PPAR $\alpha$  and  $\gamma$ .



# **I. Quercetin Protects against Linoleic Acid – Mediated Endothelial Cell Activation**

## ***Synopsis***

Consumption of plant phenolics, such as quercetin, may be associated with decreased risk of cardiovascular disease by stabilizing and protecting vascular endothelial cells against oxidative and pro-inflammatory insults. The present study focused on the effect of quercetin on linoleic acid-induced oxidative stress and the inflammatory pathways of nuclear factor- $\kappa$ B (NF- $\kappa$ B) and activator protein-1 (AP-1). Because the transcription factor peroxisome proliferator activated receptor  $\gamma$  (PPAR $\gamma$ ) has been reported to downregulate inflammatory pathways we furthermore investigated the effect of quercetin on PPAR $\gamma$ . Porcine pulmonary-arterial endothelial cells were activated with linoleic acid in the presence or absence of quercetin. Oxidative stress was markedly induced by endothelial cell exposure to linoleic acid and diminished by treatment with quercetin as measured via the oxidation of 2',7'-dichlorofluorescein. Quercetin reduced linoleic acid-mediated binding activity of NF- $\kappa$ B and AP-1 and mRNA levels of inflammatory genes, such as interleukin-6 (IL-6) and vascular cell adhesion molecule-1 (VCAM-1). Cotreatment of linoleic acid plus quercetin or vitamin E also decreased linoleic acid-induced binding activity of PPAR $\gamma$ . These data suggest that quercetin has potent antioxidative and anti-inflammatory properties and protects endothelial cells against linoleic acid-mediated cell dysfunction.

## ***Introduction***

Atherosclerotic lesions are thought to be initiated by vascular endothelial cell dysfunction. A damaged endothelium is less effective as a selectively permeable barrier to plasma components (Flarahan 1992, Ross 1999). The endothelium interacts with the blood and underlying tissues, serves as both a pro- and anti-thrombotic surface, and releases regulatory factors important in modulating vascular tone. Factors implicated in the pathogenesis of atherosclerosis include chronic and cumulative metabolic alterations

of the endothelium induced by numerous activating molecules, such as certain lipids, pro-oxidants, and inflammatory cytokines. These risk factors may contribute to an overall cellular imbalance of the oxidative stress/antioxidant balance, thus leading to chronic activation of the endothelium and alterations of the endothelial barrier function, which can result in accelerated uptake of cholesterol-rich lipoproteins into the vessel wall.

After consumption of high-energy foods, triglyceride-rich lipoproteins are elevated, and hydrolysis of triglycerides by lipoprotein lipase occurs in proximity to the endothelial surface (Zilversmit 1979). Excessive local concentration of fatty acid anions may cause endothelial injury and therefore initiate the onset of atherosclerosis. Polyunsaturated fatty acids are more susceptible to lipid peroxidation than saturated fatty acids, in particular when insufficiently protected by antioxidants. Thus, if oxidative stress is a critical underlying parameter of atherosclerosis (Steinberg and Witztum 2002), then high serum polyunsaturated fatty acid concentrations may indicate a higher risk of atherosclerosis (Kok et al. 1991).

Antioxidants, such as vitamin E, can significantly reduce the linoleic acid-mediated endothelial cell activation and loss of endothelial integrity (Hennig et al. 1996). Quercetin, as other polyphenolics, possesses high antioxidant abilities to inhibit free radical processes in cells (Lee et al. 2002), including the prevention of lipid peroxidation (Heijnen et al. 2002).

Numerous oxidative stress-sensitive transcription factors, such as nuclear factor- $\kappa$ B (NF- $\kappa$ B) and activator protein-1 (AP-1) (Turpaev 2002) can mediate an inflammatory response due to oxidative stress by inducing gene transcription of adhesion molecules and cytokines such as vascular cell adhesion molecule-1 (VCAM-1) and interleukin-6 (IL-6). Antioxidants such as quercetin could protect plasma lipids from oxidation and could therefore prevent the induction of inflammatory events. Thus quercetin could help maintain the integrity of the endothelium.

There is increasing evidence that suggests that peroxisome proliferators activated receptors (PPARs) can modulate inflammatory events and are thus anti-atherogenic (Takano 2002). For example, PPAR $\gamma$  can negatively interfere with NF- $\kappa$ B, signal transducer and activator of transcription (STAT) and AP-1 signaling pathways (Zhou and Waxman 1999, Delerive et al 1999). This could be by preventing transcription factors

from binding to their target sequences (Delerive et al 1999, Marx et al. 1999), possibly through an interaction with a subunit of the transcription factors (e.g. p65) (Delerive et al 1999). Because most of the pro-inflammatory genes are under the control of the AP-1 and NF- $\kappa$ B signaling pathways, and because PPARs can counter-regulate a wide spectrum of pro-inflammatory genes, anti-inflammatory compounds may act through PPAR signaling. PPARs binding pockets are rather promiscuous to a variety of naturally occurring lipid-like substances acting as low-affinity ligands (Van Bilsen et al. 2002). Because quercetin is a lipophilic, polyphenolic substance with a chemical structure that could potentially fit the binding pocket of PPARs, we investigated whether the anti-inflammatory properties of quercetin could be through the activation of PPAR $\gamma$ .

In this study we aimed to demonstrate that quercetin has endothelium-protective effects by preventing linoleic acid-induced oxidative stress formation and by decreasing the activation of oxidant-sensitive pathways.

## ***Materials and Methods***

### *Cell culture and experimental media*

Tissues obtained during routine slaughters were donated from the College of Agriculture, University of Kentucky. Endothelial cells were isolated from porcine pulmonary arteries by collagen digestion of extracellular matrix. The endothelial layer was transferred from tissues into culture dishes using cotton swaps (Hennig et al. 1984). Cells were subcultured in medium 199 (M-199) containing 10% (v/v) fetal bovine serum (FBS, HyClone Laboratories, Logan, UT) using standard techniques.

The experimental media were composed of M-199 enriched with 5% (v/v) FBS and fatty acids (90  $\mu$ mol/L). Fatty acids (> 99% pure) were obtained from Nu-Chek Prep (Elysian, MN). Hexane dissolved fatty acids were mixed with an equal molar amount of NaOH and dried under nitrogen to remove the solvent (Toborek et al. 2002). The sodium salt of the fatty acids was redissolved in M-199 with 5% (v/v) FBS. Thus, fatty acids were introduced into the media bound to serum albumin. Quercetin (10 – 50  $\mu$ mol/L) and vitamin E (25  $\mu$ mol/L) were added from a stock solutions in dimethylsulfoxide (DMSO) and ethanol, respectively. Controls and fatty acid groups not treated with quercetin or

vitamin E contained an equal amount of DMSO or ethanol. The final DMSO concentration in the media never exceeded 0.05% (v/v) in all treatment groups. For most experimental settings, cells were treated with quercetin and fatty acids for 6 h. Vitamin E was added 18 h prior to fatty acid treatment.

#### *Measurement of oxidative stress*

Cellular oxidation was determined by 2',7'-dichlorofluorescein (DCF) fluorescence as described earlier by Mattson *et al* (Mattson et al. 1995). This measurement of cell oxidation utilizes an imaging technique based on the conversion of 2',7'-dichlorofluorescein into fluorescent 2',7'-dichlorofluorescein as a result of activation with oxygen reactive species, primarily peroxyl radicals and peroxides. Cells were grown in 24 or 48 well plates. After treating endothelial cells with linoleic acid for 6 h, cells were washed with Hanks and loaded with 100  $\mu\text{mol/L}$  2,7-dichlorofluorescein diacetate (Molecular Probes, Inc., Eugene, OR) dissolved in Hanks by incubation for 30 minutes. Before analysis for oxidative stress, cells were washed three times in HEPES buffer. In experiments utilizing  $\text{H}_2\text{O}_2$  as an inducer of oxidative stress, 0.1  $\text{mmol/L}$   $\text{H}_2\text{O}_2$  in HEPES buffer were applied to cells after DCF-staining. Imaging studies employed a multiwell fluorescent plate reader (Molecular Devices, Sunnyvale, CA). The dye was excited at 490 nm, and emission was filtered using a 510 nm barrier filter. A blank reading (control cells without DCF staining) was subtracted from all values prior to statistical analysis.

#### *Transcription factor (NF- $\kappa\text{B}$ , AP-1 and PPAR $\gamma$ ) activation studies: electrophoretic mobility shift assay (EMSA)*

Nuclear extracts containing active proteins were prepared from cells according to the method of Dignam et al. (Dignam et al. 1993). Cells were trypsinized and pelleted by centrifugation. Cell lysis utilized the nonionic detergent Nonident-40. Lysed cells were centrifuged to pellet the cell fragments including the nucleus. Proteins were extracted with a high salt buffer containing 25% glycerol, aliquoted and flash frozen on dry ice. Nuclear extracts were incubated for 25 min with  $^{32}\text{P}$ -end-labeled oligonucleotide probes containing enhancer DNA element NF- $\kappa\text{B}$  (5' AGTTGAGGGGACTTTCCCAGGC 3'), AP-1 (5' CGCTTGATGAGTCAGCCGGAA 3') (Promega, Madison, WI) or PPAR $\gamma$  (5'

AGGTCAAAGGTCA 3') (Santa Cruz, Santa Cruz, CA). Incubation at room temperature was performed in the presence of nonspecific competitor DNA (poly dIdC). Following binding, the complexed and uncomplexed DNA in the mixture were resolved by electrophoresis in a 6.5% (w/v) non-denaturing polyacrylamide gel and visualized by autoradiography. Control reactions using 200-fold molar excess of unlabeled oligonucleotide probes or a supershift assay were performed to demonstrate the specificity of the shifted DNA-protein complexes for NF- $\kappa$ B, PPAR $\gamma$  and AP-1, respectively.

#### *IL-6 and VCAM-1 expression studies*

Total RNA was extracted from endothelial cells by the use of TRI reagent (Sigma, St. Louis, MO) according to the manufacturer's protocol. RNA concentration was determined spectrometrically reading the absorbance at 260 nm. 1  $\mu$ g RNA was reverse transcribed using the reverse transcription system kit from Promega (Madison, WI). Resulting cDNA was amplified through the polymerase chain reaction with enzyme, nucleotides and buffers purchased from Quiagen (Valencia, CA). The following primers were employed in the PCRs; IL-6 forward: 5' AAT TCG GTA CAT CCT CGA CG 3', reverse: 5' GCG CAG AAT GAG ATG AGT TG 3', VCAM-1 forward: 5' ATGACA TGC TTG AGC CAG G 3', reverse: 5' GTG TCT CCT TCT TTG ACA CT 3',  $\beta$ -actin forward: 5' GGG ACC TGA CCG ACT ACC TC 3', reverse: 5' GGG CGA TGA TCT TGA TCT TC 3'. The amplified PCR products were electrophoresed on a 2% (w/v) tris-borate EDTA agarose gel, stained with SYBR Gold (Molecular Probes, Eugene, OR) and visualized by using phosphorimaging technology (FLA-5000; Fuji, Stamford, CT).

#### *Statistical analysis*

The data were quantified and analyzed using the Scion Image and Sigma Stat software, respectively. Comparisons between treatments were made by one and two-way ANOVA with post hoc comparisons of the means made by Tukey tests. Statistical probability of  $p < 0.05$  was considered significant.

## Results

### *Quercetin attenuates linoleic acid and H<sub>2</sub>O<sub>2</sub> - induced oxidative stress*

Quercetin dose-dependently reduced oxidative stress when cells were co-treated with 90 µmol/L linoleic acid and 10, 25 or 50 µmol/L quercetin for 6 h. Significant effects were observed at 25 and 50 µmol/L quercetin (Fig. 1A). Similar effects were obtained in endothelial cells exposed to 0.1 mmol/L H<sub>2</sub>O<sub>2</sub> for to 60 min (Fig. 1B). Quercetin at the concentration of 25 and 50 µmol/L significantly reduced the generation of free radicals. Increasing the concentration of quercetin to 50 µmol/L did not show additional protection against free radical formation compared to 25 µmol/L quercetin.

### *Quercetin decreases linoleic acid - induced binding activity of NF-κB and AP-1*

Linoleic acid increased DNA binding activity of both transcription factors NF-κB and AP-1 as determined by EMSA. Cotreatment of quercetin and linoleic acid for 6 hours down-regulated the activation of NF-κB and AP-1 (Figs. 2 and 3, respectively). Consistent with the observations made with measuring oxidative stress, 25 µmol/L appeared to be the most effective concentration of quercetin with no additional benefit of higher concentrations to down-regulate NF-κB (Fig. 2). Maximal downregulation of linoleic acid-induced AP-1 binding activity already occurred at 10 µmol/L quercetin (Fig. 3).

### *Quercetin protects against linoleic acid-induced IL-6 and VCAM-1 gene expression*

VCAM-1 expression was upregulated after a 6 h exposure to linoleic acid (Fig. 4) and co-exposure to quercetin protected against this effect. The quercetin-induced decrease in VCAM-1 gene expression was concentration dependent with complete blockage of linoleic acid induced gene expression in the presence of 25 µmol/L quercetin (Fig. 4).

Cytokine IL-6 expression was upregulated in response to linoleic acid treatment (Fig. 5). Quercetin was less potent in down-regulating IL-6 mRNA compared to the effects seen in the down-regulation of NF-κB and AP-1 binding activity or VCAM-1 mRNA. Indeed, quercetin was only effective if applied in higher concentrations (50 µmol/L).

*Quercetin and vitamin E decrease linoleic acid-induced PPAR $\gamma$  binding activity*

PPAR $\gamma$  is known to protect cells against proinflammatory and prooxidative insults, and linoleic acid is a natural ligand for this transcription factor. Therefore the effects of linoleic acid and/or quercetin on PPAR $\gamma$  DNA binding activity were assessed in the present study. Exposure to linoleic acid for 6 h markedly induced PPAR $\gamma$  activity (Figs. 6 and 7). Although quercetin alone did not affect binding of this transcription factor, it diminished PPAR $\gamma$  activation in cells cotreated with linoleic acid. To assess if the effects of quercetin on activation of PPAR $\gamma$  were specific, cells were pretreated with another antioxidant, vitamin E (25  $\mu$ mol/L for 18 h) and treated with linoleic acid for 6 h. Similar to the effects exerted by quercetin, vitamin E also protected against linoleic acid-induced binding activity of PPAR $\gamma$  (Fig. 7).

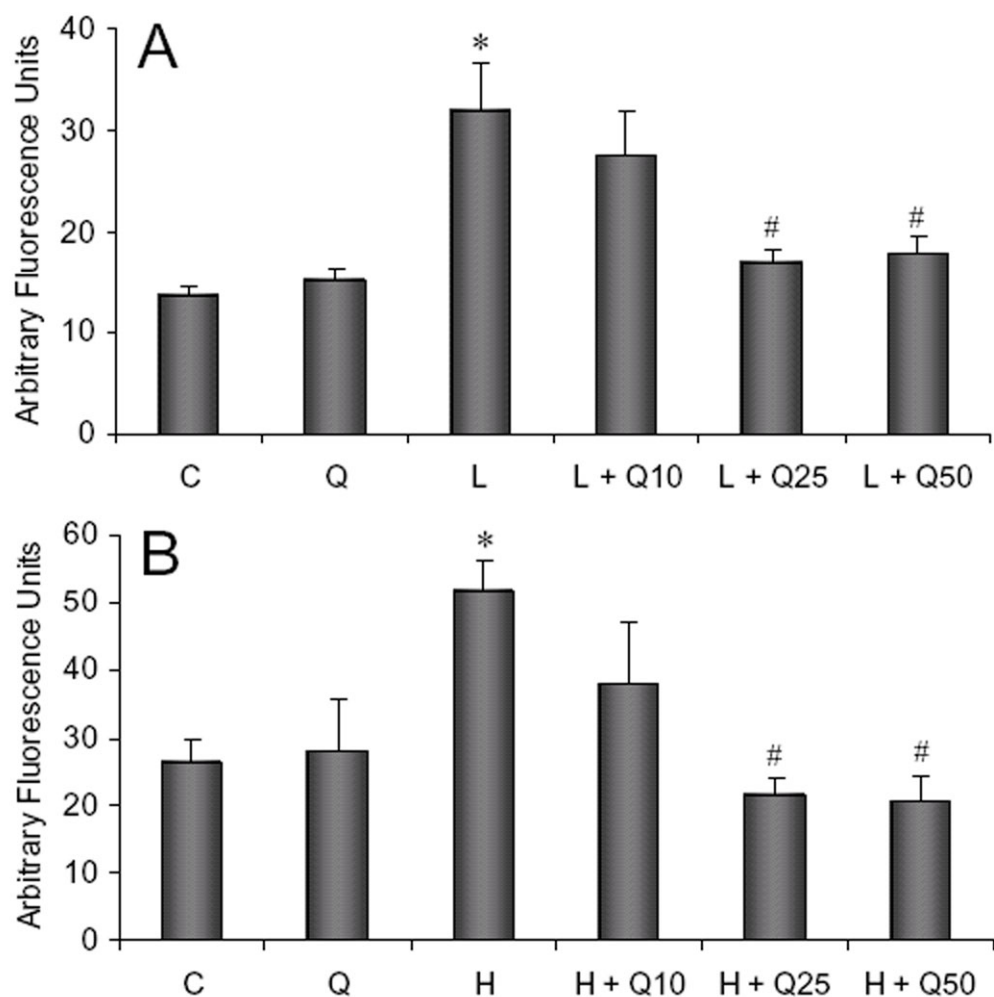


Figure 1, Quercetin decreases oxidative stress in endothelial cells

Fatty acid (Fig. 1A) and  $H_2O_2$  (Fig. 1B) induced oxidative stress in primary endothelial cells. Cells were grown in 24 well plates, exposed to 90  $\mu\text{mol/L}$  fatty acid and/or various concentrations of quercetin for 6 h. Oxidative stress was determined spectrometrically by measuring the conversion of dichlorofluorescein into dichlorofluorescein. Q, quercetin; L, linoleic acid; H,  $H_2O_2$ ; concentrations in  $\mu\text{mol/L}$ . Values are means  $\pm$  SEM ( $n = 3$ ).

\*Significantly different as compared to control. # Values in the linoleic acid plus quercetin (L + Q) and  $H_2O_2$  plus quercetin (H + Q) groups are significantly different as compared to the linoleic acid (L) group.



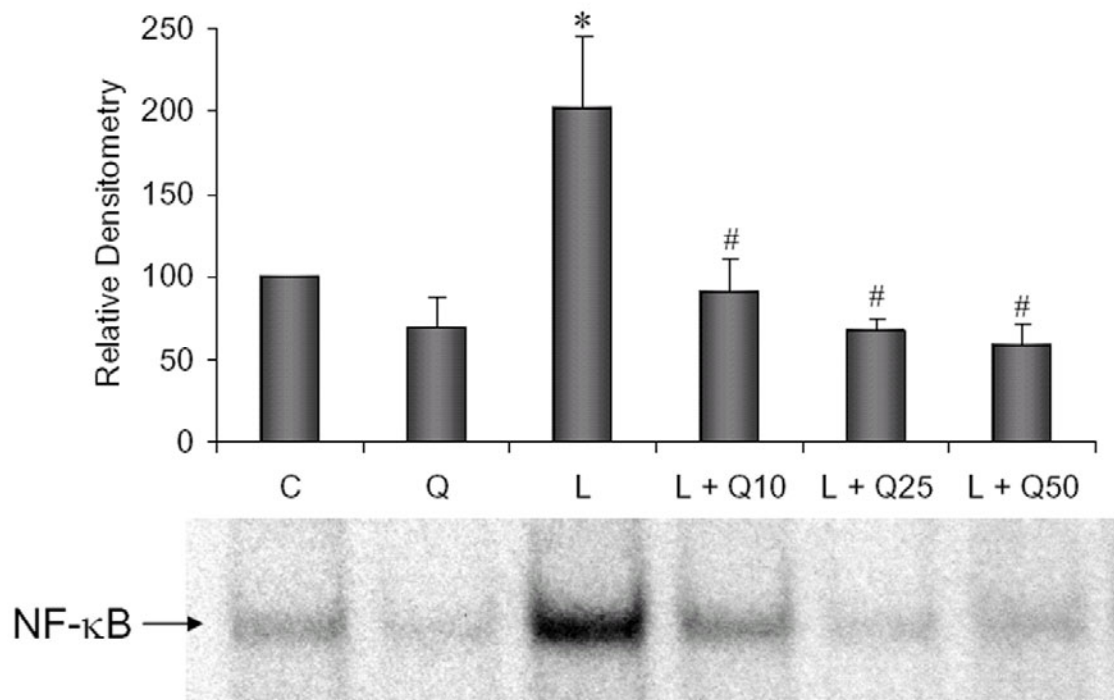


Figure 2, Quercetin prevents NF- $\kappa$ B DNA binding

Fatty acid-induced NF- $\kappa$ B binding activity in endothelial cells. Primary endothelial cells were cotreated with 90  $\mu$ mol/L linoleic acid and 10, 25 or 50  $\mu$ mol/L quercetin for 6 h. The binding activity of the transcription factor was determined by electrophoresis mobility shift assay (EMSA). Q, quercetin; L, linoleic acid; concentrations in  $\mu$ mol/L. Values are means  $\pm$  SEM (n = 3). \*Significantly different compared to control. # Values in the linoleic acid plus quercetin (L + Q) groups are significantly different as compared to the linoleic acid (L) group.

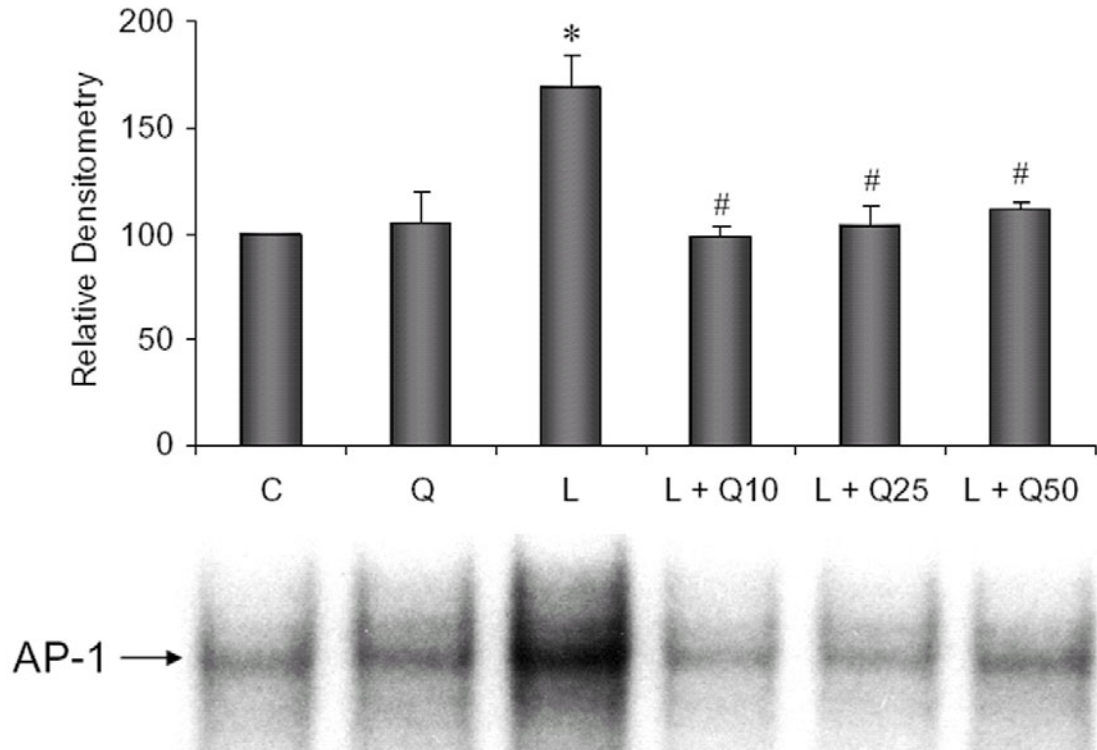


Figure 3, Quercetin prevents AP-1 DNA binding

Fatty acid-induced AP-1 binding activity in endothelial cells. Primary endothelial cells were cotreated with 90  $\mu\text{mol/L}$  linoleic acid and 10, 25 or 50  $\mu\text{mol/L}$  quercetin for 6 h. The binding activity of the transcription factor was determined by electrophoresis mobility shift assay (EMSA). Q, quercetin; L, linoleic acid; concentrations in  $\mu\text{mol/L}$ . Values are means  $\pm$  SEM ( $n = 3$ ). \*Significantly different compared to control. # Values in the linoleic acid plus quercetin (L + Q) groups are significantly different as compared to the linoleic acid (L) group.

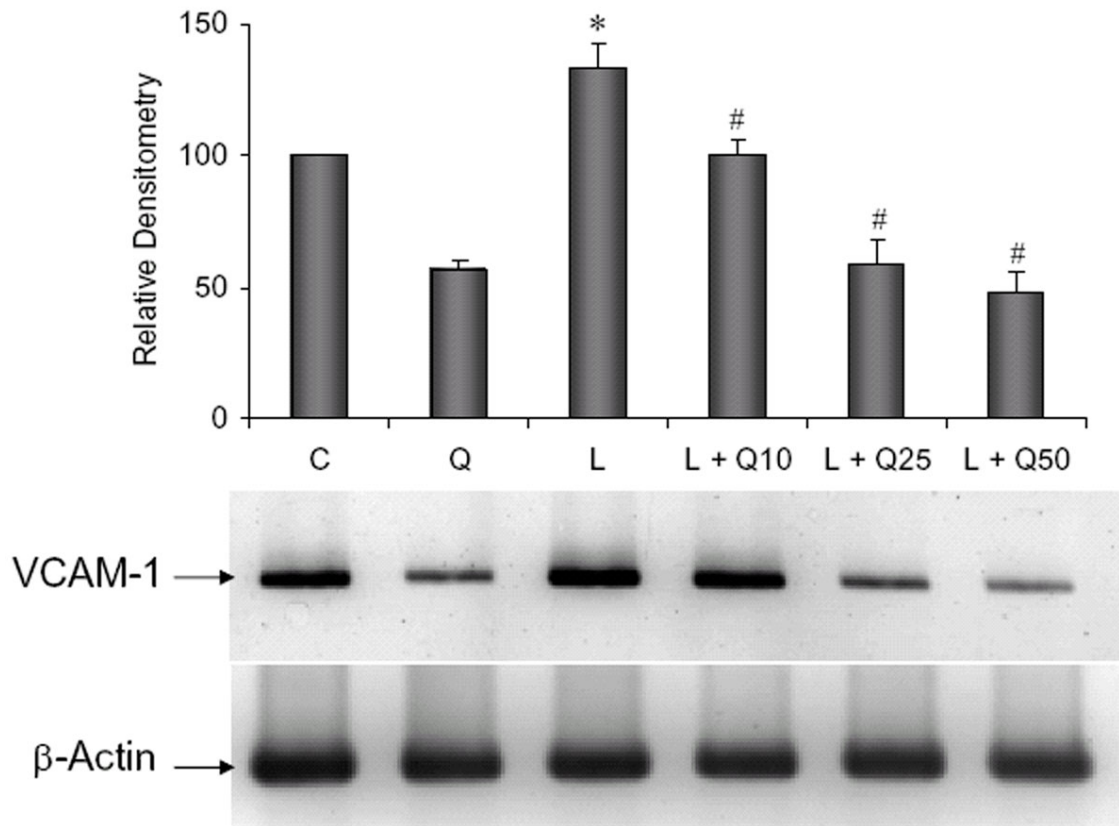


Figure 4, Quercetin reduces VCAM-1 mRNA expression

Effects of linoleic acid and quercetin on VCAM-1mRNA levels in endothelial cells as measured by reverse transcriptase–polymerase chain reaction (RT-PCR). Endothelial cells were exposed to linoleic acid and/or quercetin for 6 h.  $\beta$ -actin was used as a housekeeping gene. Q, quercetin; L, linoleic acid; concentrations in  $\mu\text{mol/L}$ . Values are means  $\pm$  SEM ( $n = 3$ ).

\*Significantly different compared to control. # Values in the linoleic acid plus quercetin (L + Q) group(s) are significantly different as compared to the linoleic acid (L) group.

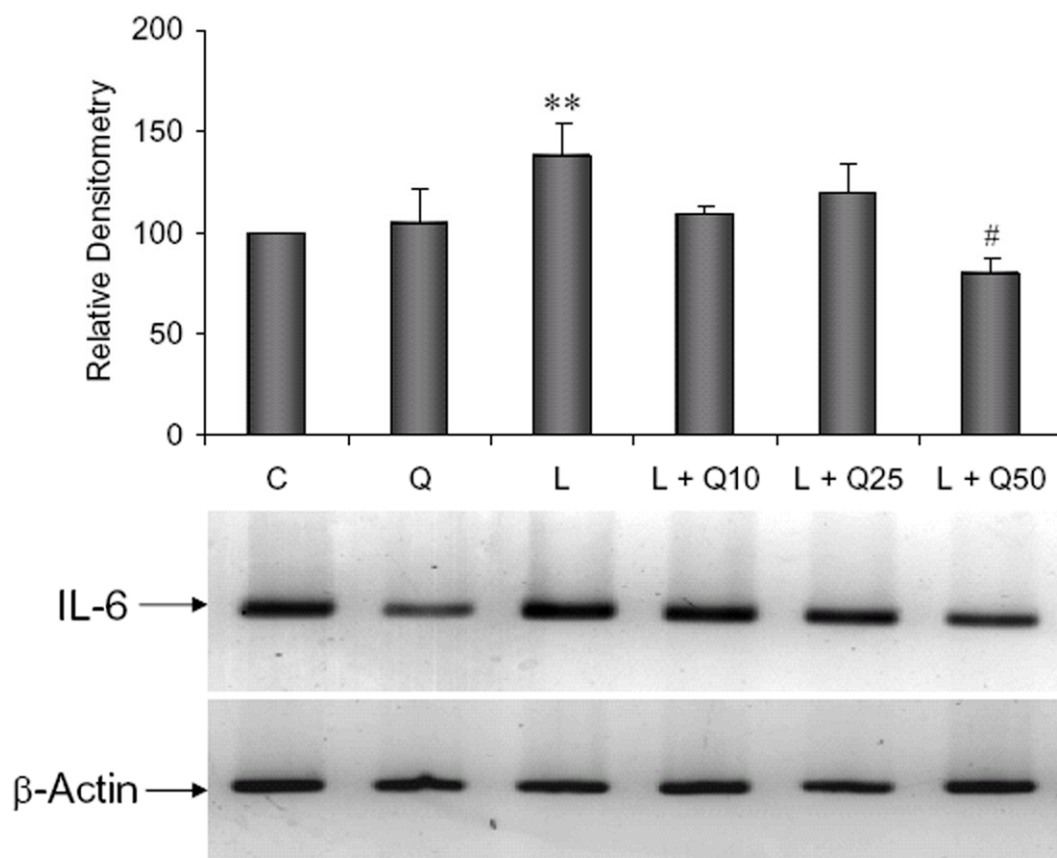


Figure 5, Quercetin reduces IL-6 mRNA expression

Effects of linoleic acid and quercetin on IL-6 mRNA levels in endothelial cells as measured by reverse transcriptase–polymerase chain reaction (RT-PCR). Endothelial cells were exposed to linoleic acid and/or quercetin for 6 h.  $\beta$ -actin was used as a housekeeping gene. Q, quercetin; L, linoleic acid; concentrations in  $\mu\text{mol/L}$ . Values are means  $\pm$  SEM ( $n = 3$ ).

# Values in the linoleic acid plus quercetin (L + Q) group(s) are significantly different as compared to the \*\* linoleic acid (L) group.

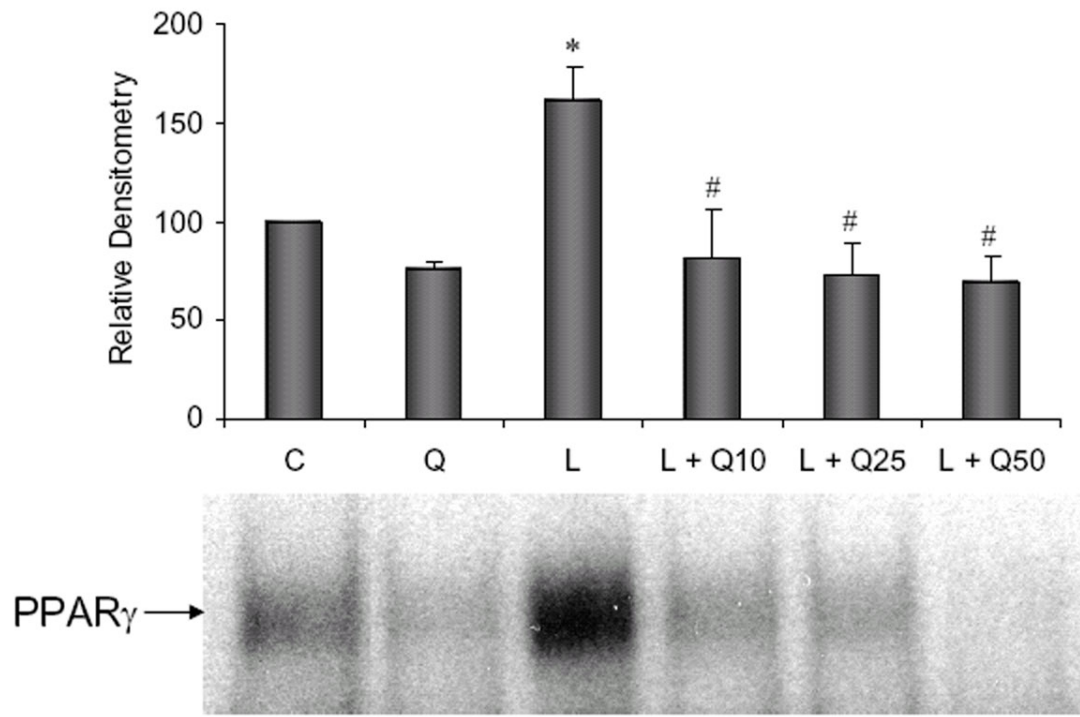


Figure 6, Quercetin inhibits PPAR DNA binding

Effects of linoleic acid and quercetin on peroxisome proliferator activated receptor gamma (PPAR $\gamma$ ) activity in endothelial cells. Primary endothelial cells were cotreated with 90  $\mu$ mol/L linoleic acid and 10, 25 or 50  $\mu$ mol/L quercetin for 6 h. The binding activity of the transcription factor was determined by EMSA. Q, quercetin; L, linoleic acid; concentrations in  $\mu$ mol/L. Values are means  $\pm$  SEM (n = 3). \*Significantly different compared to control. # Values in the linoleic acid plus quercetin (L + Q) group(s) are significantly different as compared to the linoleic acid (L) group.

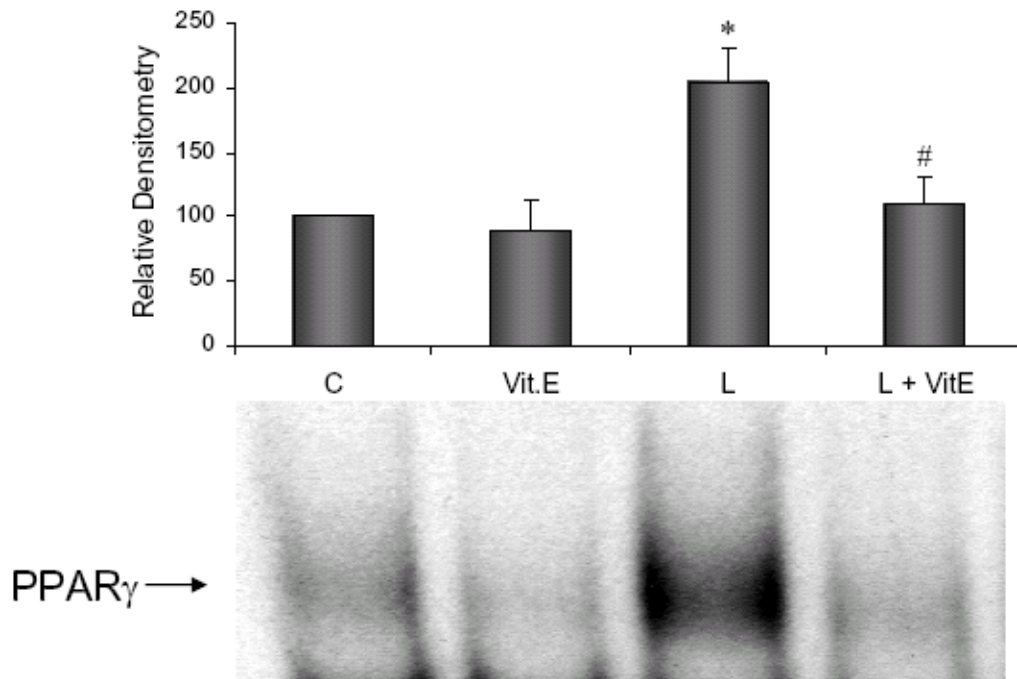


Figure 7, Vitamin E inhibits PPAR DNA binding

Effects of linoleic acid and vitamin E on peroxisome proliferator activated receptor gamma (PPAR $\gamma$ ) activity in endothelial cells. Primary endothelial cells were pretreated with 25  $\mu$ mol/L vitamin E 18 hours prior to treatment with 90  $\mu$ mol/L linoleic acid for 6 h. The binding activity of the transcription factor was determined by EMSA. L, linoleic acid; concentrations in  $\mu$ mol/L. Values are means  $\pm$  SEM (n = 3). \*Significantly different compared to control. # Values in the linoleic acid plus vitamin E (L + VitE) group is significantly different as compared to the linoleic acid (L) group.

## Discussion

Foods rich in plant phenolics may be associated with a decreased risk of atherosclerosis by protecting vascular endothelial cells against proinflammatory lipids. In the present study we demonstrated an overall antioxidant and antiinflammatory effect of quercetin. We have reported before (Hennig et al. 2000) that linoleic acid markedly increases oxidative stress in cultured endothelial cells as measured by DCF fluorescence. Here we show that cotreatment with quercetin significantly inhibits the formation of reactive oxygen species (ROS) after treating endothelial cells with linoleic acid. The reduction in the generation of ROS is likely due to a direct scavenging effect of quercetin. The chemical structure of quercetin allows the prediction of antioxidant properties of this molecule. It has been suggested that quercetin gets oxidized to a semiquinone and quinone (Metodiewa et al. 2001) while neutralizing ROS. Both the semiquinone and the quinone could be regenerated by glutathione, vitamins E and C (Metodiewa et al. 2001). Therefore, quercetin could be a valuable contributor to the cellular oxidant defense system. On the other hand, when applied in high concentrations and for long incubation times, quercetin was shown to exhibit prooxidant effects, as reported by others (Metodiewa et al. 2001, Long et al. 2000) and as was also observed by us upon extended treatment with high levels of this phenolic compound (data not shown). As a consequence, the antioxidant capacities of quercetin have been challenged. However, a prooxidant effect of quercetin *in vivo* seems to be unlikely as plasma quercetin levels do not reach concentrations that induce oxidative stress in *in vitro* experiments (Hollman and Katan 1997).

The antioxidant properties of quercetin also could be responsible in part for its antiinflammatory effects observed in the present study. We showed that quercetin can down-regulate the linoleic acid-induced activation of both NF- $\kappa$ B and AP-1. These are oxidative stress-sensitive transcription factors and can therefore be modified by oxidants and antioxidants. The precise way by which linoleic acid induces an inflammatory response in endothelial cells is not clear. However, oxidation products of linoleic acid might be directly involved in the activation of NF- $\kappa$ B. Lipid peroxides rather than H<sub>2</sub>O<sub>2</sub> have been suggested to mediate the activation of NF- $\kappa$ B in response to oxidative stress (Bowie and O'Neill 2000). It is possible that lipid metabolites and derivatives, including

oxidized fatty acids, can induce the inflammation, in addition to the native linoleic acid itself. Furthermore, quercetin has been suggested to inhibit I $\kappa$ B kinase (Peet and Li 1999) and c-Jun kinase (Yoshizumi et al. 2002), which subsequently could lead to suppression of NF- $\kappa$ B and AP-1 activation. Activation of NF- $\kappa$ B leads to expression of inflammatory cytokines and adhesion molecules, resulting in recruitment of monocytes and accelerated development of atherosclerosis (Sluiter et al. 1993). The fact that most of the pro-inflammatory genes are under the control of the AP-1 and NF- $\kappa$ B signaling pathways (Valen et al. 2001) and that quercetin can counter-regulate these pathways implies the potent anti-inflammatory properties of quercetin. In support of its anti-inflammatory properties, we demonstrated that quercetin can block the linoleic acid-induced expression of both VCAM-1 and IL-6, a down-stream event of NF- $\kappa$ B activation (Li and Stark 2002).

Because PPAR $\gamma$  can negatively interfere with NF- $\kappa$ B, STAT and AP-1 signaling pathways (Zhou and Waxman 1999, Delerive et al 1999), we further investigated whether quercetin can affect PPAR $\gamma$  binding activity. Even though both PPAR $\alpha$  and PPAR $\gamma$  have been reported to be anti-inflammatory and anti-atherogenic, PPAR $\alpha$  is involved in lipid metabolism and could therefore be activated independently of fatty acid modification by oxidation. Thus, the present study focuses on PPAR $\gamma$ . Although quercetin has a chemical structure that could potentially be a ligand for PPAR $\gamma$ , exposure to quercetin alone did not activate this transcription factor. Consistent with the known overall effects of fatty acids on PPAR $\gamma$  activation, treatment with linoleic acid induced PPAR $\gamma$  binding activity in endothelial cells. However, cotreatment with quercetin markedly downregulated linoleic acid-induced PPAR $\gamma$  activation. These results are consistent with earlier reports which showed that quercetin can mediate downregulation of PPAR $\gamma$  in transiently transfected macrophages (Liang et al. 2001) and in murine epidermal keratinocytes (Thuillier et al. 2002). Because linoleic acid can induce oxidative stress and inflammation and at the same time activate PPAR, we suspected that lipid oxidation is involved in activating the PPAR pathway. To further address the question whether decreased PPAR $\gamma$  activation observed in the present study are specific for quercetin, endothelial cells were treated with another antioxidant (vitamin E) followed by exposure to linoleic acid. Vitamin E also prevented the linoleic acid-mediated activation of



PPAR $\gamma$ . These data suggest that quercetin can reduce the linoleic acid – mediated activation of PPAR $\gamma$  due to its antioxidant effect and the prevention of lipid oxidation. Previous studies indicate that oxidized products of linoleic acid are good ligands for PPARs (Bull et al. 2003). Quercetin may act as an antioxidant and thus the decreased formation of oxidized products of linoleic acid might be a reason for the lower binding of linoleic acid to PPAR $\gamma$  in the presence of quercetin.

These data suggest that the observed quercetin-mediated protection against linoleic acid-induced endothelial cell activation is independent of PPAR $\gamma$  signaling. In addition, it appears that the diminished PPAR $\gamma$  DNA binding activity observed in cells exposed to linoleic acid plus quercetin may be related to a general antioxidant effects of this polyphenolic. Indeed, PPARs may act as critical rescue molecules, by downregulating oxidative stress-sensitive and inflammatory signaling pathways. In cells that are protected by antioxidants such as quercetin and vitamin E, oxidative stress sensitive pathways, including PPAR $\gamma$ , are less likely to become activated.

Overall, our data suggest that quercetin is a potent antioxidant and anti-inflammatory substance, which can protect the endothelium against oxidative stress and inflammatory events, despite downregulation of PPAR $\gamma$  activation. Specifically, quercetin inhibited linoleic acid-induced activation of oxidative stress-sensitive pathways, such as NF- $\kappa$ B and AP-1, and inflammatory genes, such as VCAM-1 and IL-6. These data support the hypothesis that plant phenolics like quercetin can help prevent the development of atherosclerosis by down-regulating the expression of inflammatory cytokines and adhesion molecules.

## **II. PPAR $\alpha$ and $\gamma$ Require Zinc for their Protective Properties in Porcine Endothelial Cells**

### ***Synopsis***

Zinc is an essential structural component of various proteins and crucial for the integrity of the vascular endothelium. The present study focused on the effect of zinc deficiency on the anti-inflammatory properties of peroxisome proliferator activated receptor (PPAR)  $\alpha$  and  $\gamma$  agonists. Porcine pulmonary-arterial endothelial cells were deprived from zinc by chelator N,N,N',N'-tetrakis (2-pyridylmethyl)-ethylene diamine (TPEN). Cells were exposed to tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) for 2 h following pretreatment with the PPAR $\alpha$  agonists fenofibrate or ciprofibrate or the PPAR $\gamma$  agonists thiazolidinedione or troglitazone. The inflammatory response was tested by measuring nuclear factor-kappaB (NF- $\kappa$ B) and activator protein-1 (AP-1) binding activities as well as by measuring mRNA expression levels of inflammatory genes, such as vascular cell adhesion molecule-1 (VCAM-1) and interleukin-6 (IL-6). All PPAR agonists tested lost their potency to downregulate the TNF- $\alpha$  induced inflammatory response in zinc deficient cells. However, if zinc was added back, all PPAR agonists significantly downregulated the TNF- $\alpha$  mediated induction of inflammatory transcription factors NF- $\kappa$ B and AP-1 and significantly reduced the expression of their target genes VCAM-1 and IL-6. We therefore hypothesize that zinc is required for the PPAR $\alpha$  and  $\gamma$  DNA binding activity. Indeed, zinc deficiency significantly reduced the agonist-induced binding activity of PPAR $\alpha$  and  $\gamma$  to the PPAR response element (PPRE). Our data demonstrate the importance of zinc in PPAR signaling and the requirement of zinc for the anti-inflammatory properties of PPAR $\alpha$  and  $\gamma$  agonists.

### ***Introduction***

Atherosclerotic lesions are thought to be initiated by vascular endothelial cell dysfunction. Factors implicated in the pathogenesis of atherosclerosis include chronic

and cumulative metabolic alterations of the endothelium induced by numerous activating molecules, such as lipids, pro-oxidants, and inflammatory cytokines. These risk factors induce certain cell signaling pathways leading to the activation of proinflammatory transcription factors such as nuclear factor- $\kappa$ B (NF- $\kappa$ B) and activator protein-1 (AP-1) (Hennig 2001). NF- $\kappa$ B and AP-1 control most adhesion molecules and cytokines like vascular cell adhesion molecule-1 (VCAM-1) and interleukin-6 (IL-6) in endothelial cells. The expression of cytokines and adhesion molecules further enhances the inflammation by recruiting monocytes and facilitating their binding to and migration through the endothelium. This loss of the endothelial barrier function can increase the formation of foam cells in the subendothelial space, subsequently leading to the formation of fatty streaks.

PPAR $\alpha$  and  $\gamma$  agonists have been shown to be protective against these events by downregulating underlying proinflammatory signaling pathways. PPARs have been shown to negatively interfere with NF- $\kappa$ B, signal transducer and activator of transcription (STAT) and AP-1 signaling pathways (Zhou and Waxman 1999, Delerive et al. 1999) and can therefore prevent the expression of inflammatory genes such as adhesion molecules and cytokines. Indeed, clinical and experimental evidence suggests that PPAR activation decreases the incidence of cardiovascular diseases. PPARs appear to act at least in part directly at the level of the vascular wall in addition to correcting metabolic disorders (Torra et al. 2001).

However, the precise mechanisms by which PPARs can inhibit inflammatory transcription factors are not clear. In addition to ligand-dependent regulation, it was demonstrated that the transcriptional activity of PPARs could be altered by covalent modifications such as phosphorylation (Hu et al. 1996, Camp and Tafuri 1997). Furthermore, the activity of nuclear receptors can be influenced by cofactors which modulate signaling and interactions with the basal transcription machinery (Robyr et al. 2000). So far, only a few studies addressed the interactions between PPARs and cofactors, and none of them included trace elements.

We propose that zinc could be an important factor for the antiinflammatory function of PPARs. Our laboratory has substantial data on the protective properties of zinc against inflammatory and pro-oxidative stimuli such as TNF- $\alpha$  or linoleic acid (Hennig et al.

1999, Connell et al. 1997, Meerarani et al. 2000). Zinc can stabilize the vascular endothelium (Hennig et al. 1992), and protect against TNF-induced disruption of endothelial barrier function (Hennig et al. 1993). However, the protective properties of zinc are not as well understood in inflammatory pathways that are directly induced by a signaling cascade triggered by TNF- $\alpha$  where oxidative stress is not the main inducer of inflammatory transcription factors. Little is known about the requirements and functions of zinc in maintaining the integrity of the vasculature and particularly the vascular endothelium. Zinc might play an important role in endothelial protection against pro-oxidative and pro-inflammatory insults which are critical events in early pathogenesis of atherosclerosis.

In this study we aimed to demonstrate that zinc is required for the antiinflammatory properties of PPAR $\alpha$  and  $\gamma$  and that activation of PPAR $\alpha$  or  $\gamma$  is defective in zinc deficient cells.

## **Materials and Methods**

### *Cell culture and experimental media*

Endothelial cells were isolated from porcine pulmonary arteries as described previously (Toborek et al. 2002). Arteries obtained during routine slaughter were donated from the College of Agriculture, University of Kentucky. Cells were subcultured in medium 199 (M-199) containing 10% (v/v) fetal bovine serum (FBS, HyClone Laboratories, Logan, UT) using standard techniques.

The experimental media were composed of M-199 enriched with 5% (v/v) FBS. Zinc (20  $\mu$ mol/L) was added as zinc acetate from a stock solution in water. TPEN (2  $\mu$ mol/L) and PPAR agonists (10 – 25  $\mu$ mol/L) were added from a stock solutions in ethanol and dimethylsulfoxide (DMSO), respectively. TNF $\alpha$  (0.01 ng/ml) was added directly into the culture media 2 h prior to termination of the treatment. Unless otherwise stated, chemicals were purchased from Sigma, St. Louis, MO. All treatment groups contained an equal amount of DMSO or ethanol. The final DMSO concentration in the media never exceeded 0.05% (v/v) in all treatment groups. For most experimental settings, cells were treated with zinc and/or TPEN for 24 h, PPAR agonists for 8 -18 h and TNF for 2 h.

*Transcription factor (NF- $\kappa$ B, AP-1 and PPAR $\gamma$ ) activation studies: electrophoretic mobility shift assay (EMSA)*

Nuclear extracts containing active proteins were prepared from cells according to the method of Dignam et al. (Dignam et al. 1993). Nuclear extracts were incubated for 25 min with  $^{32}$ P-end-labeled oligonucleotide probes containing enhancer DNA element NF- $\kappa$ B (5' AGTTGAGGGGACTTTCCCAGGC 3'), AP-1 (5' CGCTTGATGAGTCAGCCGGAA 3') (Promega, Madison, WI) or PPAR $\gamma$  (5' AGGTCAAAGGTCA 3') (Santa Cruz, Santa Cruz, CA). Incubation at room temperature was performed in the presence of nonspecific competitor DNA. Following binding, the complexed and uncomplexed DNA in the mixture were resolved by electrophoresis in a 6.5% (w/v) non-denaturing polyacrylamide gel and visualized by autoradiography. Control reactions using supershift assay were performed to demonstrate the specificity of the shifted DNA-protein complexes for NF- $\kappa$ B, PPAR $\gamma$  and AP-1, respectively. All antibodies used were obtained from Santa Cruz, Santa Cruz, CA).

*IL-6 and VCAM-1 expression studies*

Total RNA was extracted from endothelial cells by the use of TRI reagent (Sigma, St. Louis, MO) according to the manufacturer's protocol. Gene expression was determined through reverse transcription – polymerase chain reaction (RT-PCR) as described earlier (Lee et al. 2001). The following primers were employed in the PCRs; IL-6 forward: 5' AAT TCG GTA CAT CCT CGA CG 3', reverse: 5' GCG CAG AAT GAG ATG AGT TG 3', VCAM-1 forward: 5' ATGACA TGC TTG AGC CAG G 3', reverse: 5' GTG TCT CCT TCT TTG ACA CT 3',  $\beta$ -actin forward: 5' GGG ACC TGA CCG ACT ACC TC 3', reverse: 5' GGG CGA TGA TCT TGA TCT TC 3'. The amplified PCR products were electrophoresed on a 2% (w/v) tris-borate EDTA agarose gel, stained with SYBR Gold (Molecular Probes, Eugene, OR) and visualized by using phosphorimaging technology (FLA-5000; Fuji, Stamford, CT).

### *Statistical analysis*

The data were quantified and analyzed using the Scion Image and Sigma Stat software, respectively. Comparisons between treatments were made by one way ANOVA with post hoc comparisons of the means made by Tukey tests. Statistical probability of  $p < 0.05$  was considered significant.

## **Results**

### *PPAR agonists and zinc downregulate the TNF $\alpha$ - induced binding activity of NF- $\kappa$ B and AP-1*

TNF- $\alpha$  increased DNA binding activity of the transcription factors AP-1 and was further increased by zinc chelation with TPEN (Fig. 1). In the zinc deficient cells neither the PPAR $\gamma$  agonist TZD (25  $\mu$ mol/L) nor the PPAR $\alpha$  agonist FF (10  $\mu$ mol/L) significantly downregulated the TNF- $\alpha$  mediated AP-1 induction ( $p > 0.2$ ). In cells that received zinc both PPAR agonists significantly decreased the binding activity of AP-1. Similar results were obtained using CF (25  $\mu$ mol/L) and TG (25  $\mu$ mol/L) as alternative agonists for PPAR $\alpha$  and  $\gamma$  respectively (data not shown). Furthermore, TNF- $\alpha$  remarkably increased the DNA binding activity of NF- $\kappa$ B in zinc deficient cells (Fig. 2). Addition of either PPAR agonists TZD, CF, TG or FF (only selected data are shown) or zinc ameliorated the NF- $\kappa$ B activation in zinc deficient cells ( $p > 0.3$ ). However, a significant downregulation could only be observed in zinc sufficient cells treated with PPAR agonists.

### *PPAR agonists and zinc protect against TNF- $\alpha$ -induced IL-6 and VCAM-1 gene expression*

IL-6 and VCAM-1 expression was upregulated after a 2 h exposure to TNF- $\alpha$  and was significantly upregulated in zinc depleted cells exposed to TNF- $\alpha$  (Fig. 3A and 3B). When administered to zinc deficient cells, neither zinc nor PPAR $\gamma$  agonist TZD significantly downregulated VCAM-1 and IL-6 gene expression ( $p > 0.4$ ). TPEN alone however, did not induce VCAM-1 expression. However, when zinc and TZD were added

in concert to zinc deficient cells, IL-6 and VCAM-1 mRNA levels were significantly decreased.

*PPAR agonists induced PPAR binding activity only in zinc sufficient cells*

PPAR $\gamma$  agonist TZD and PPAR $\alpha$  agonist CF both induced binding to the PPRE as determined by EMSA (Figs. 4A and 4B). Zinc depletion by TPEN alone did not affect binding to the PPRE. However, cotreatment of cells with TPEN and PPAR agonists resulted in a significant decrease in the PPAR DNA binding activity.

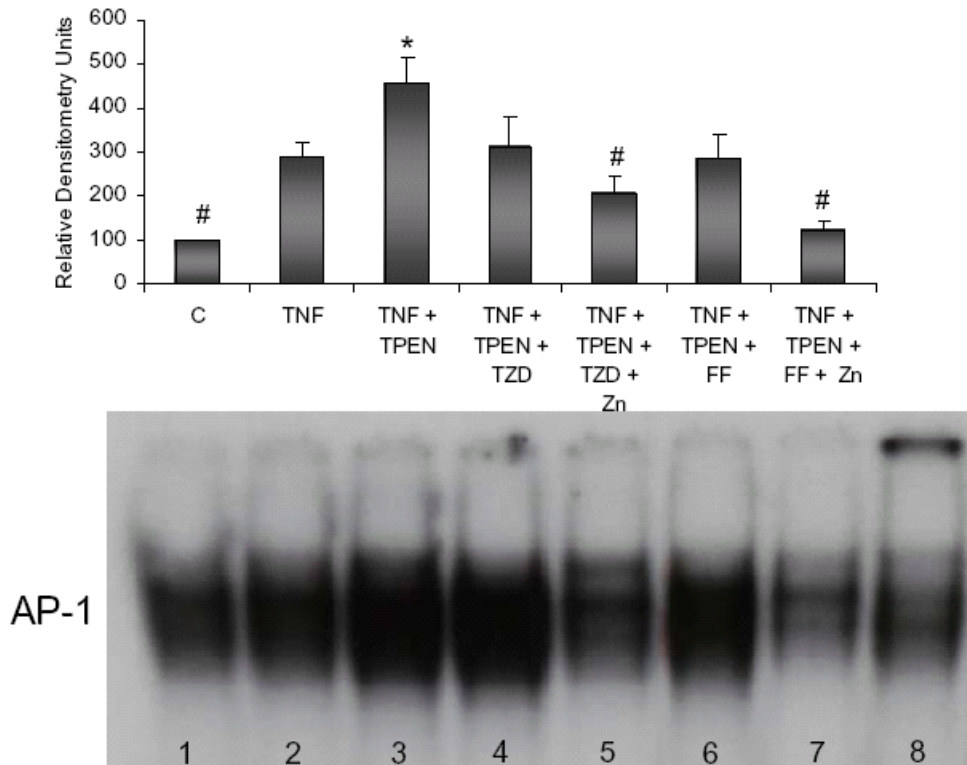


Figure 8, PPAR agonists require zinc to prevent AP-1 DNA binding

TNF- $\alpha$  mediated induction of AP-1 in primary endothelial cells. Cells were deprived from zinc by chelation with 2  $\mu\text{mol/L}$  TPEN. Selected treatment groups received 20  $\mu\text{mol/L}$  zinc and/or PPAR $\alpha$  agonist fenofibrate (10  $\mu\text{mol/L}$ ) or  $\gamma$  agonist thiazolidinedione (25  $\mu\text{mol/L}$ ) prior to exposure to TNF. Lane 8 shows a super shift that confirms the depicted lane to be that of AP-1. TNF, tumor necrosis factor  $\alpha$ ; TPEN, N,N,N',N'-tetrakis (2-pyridylmethyl)-ethylene diamine; Zn, zinc; TZD, thiazolidinedione; FF, fenofibrate; concentrations in  $\mu\text{mol/L}$ . Values are means  $\pm$  SEM ( $n = 3$ ). \* Significantly different as compared to control and treatment groups as marked (#).



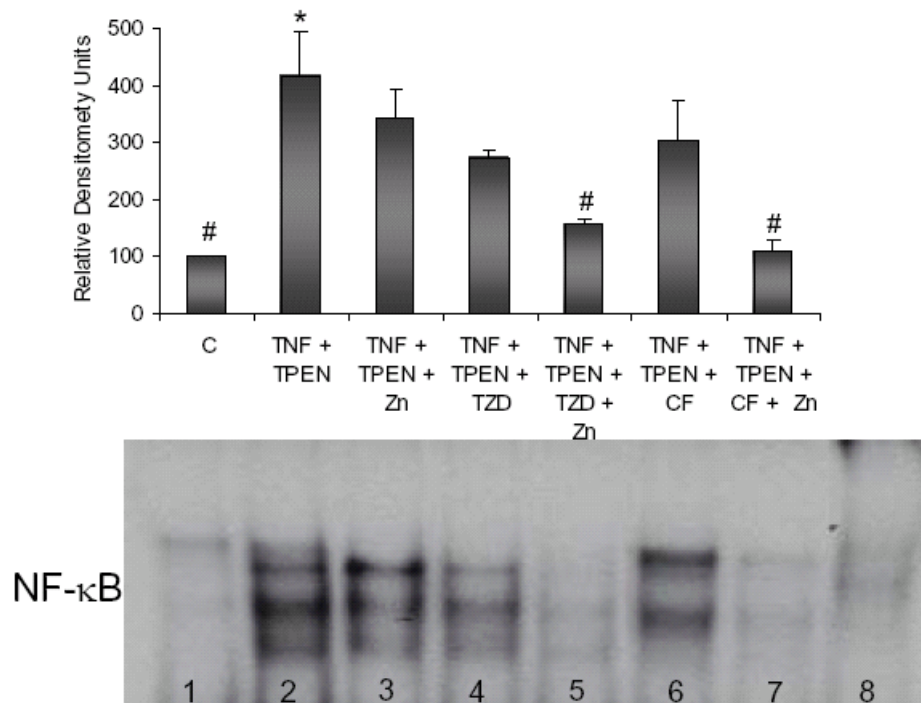


Figure 9, PPAR agonists require zinc to prevent NF- $\kappa$ B DNA binding

TNF- $\alpha$  mediated induction of NF- $\kappa$ B in porcine endothelial cells. Cells were deprived from zinc by chelation with 2  $\mu$ mol/L TPEN. Selected treatment groups received 20  $\mu$ mol/L zinc and/or treated with PPAR $\alpha$  agonist ciprofibrate (25  $\mu$ mol/L) or  $\gamma$  agonist thiazolidinedione (25  $\mu$ mol/L) prior to exposure to TNF- $\alpha$ . Lane 8 shows a super shift that confirms the depicted lane to be that of NF- $\kappa$ B. TNF, tumor necrosis factor  $\alpha$ ; TPEN, N,N,N',N'-tetrakis (2-pyridylmethyl)-ethylene diamine; Zn, zinc; TZD, thiazolidinedione; CF, ciprofibrate; concentrations in  $\mu$ mol/L. Values are means  $\pm$  SEM (n = 3). \*Significantly different as compared to control and treatment groups as marked (#).

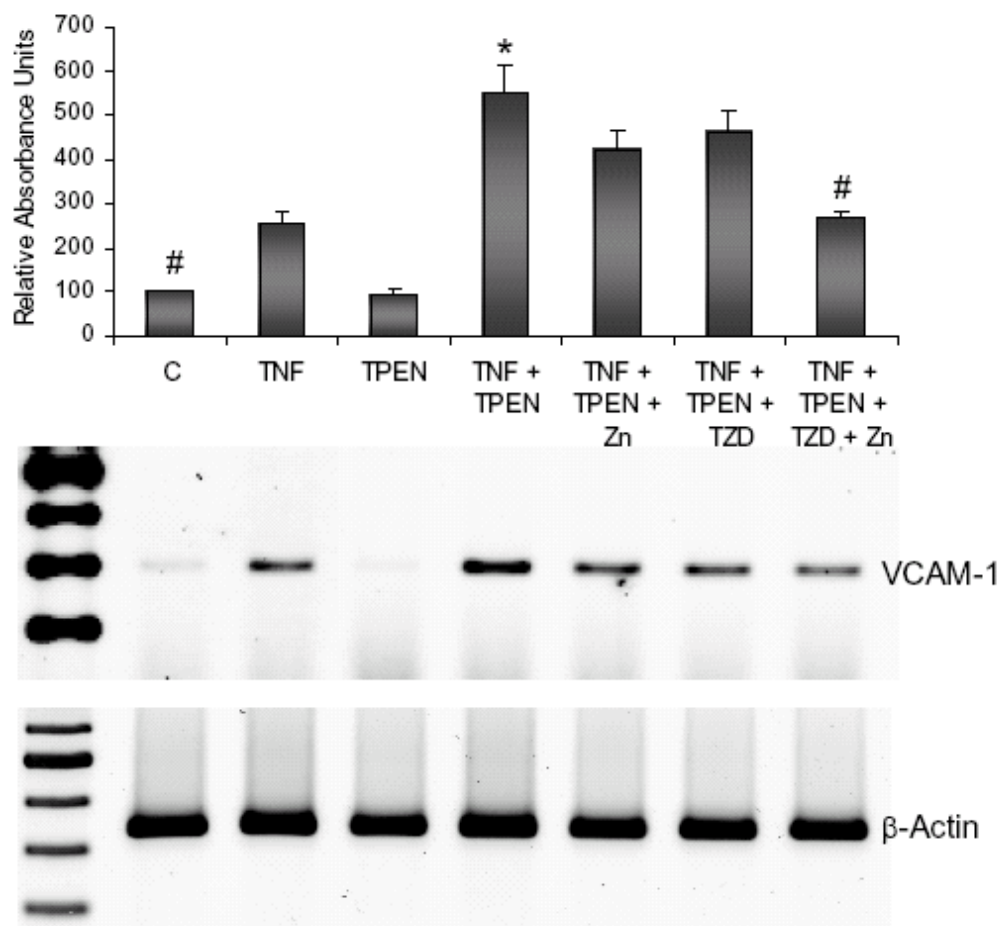


Figure 10, PPAR agonists require zinc to prevent VCAM-1 mRNA expression

Effects of TNF- $\alpha$ , zinc depletion/supplementation and PPAR agonists on VCAM-1 mRNA levels in endothelial cells. Endothelial cells were pretreated with PPAR agonists and zinc/zinc chelator TPEN (18 h) and exposed to TNF- $\alpha$  for 2 h.  $\beta$ -actin was used as a housekeeping gene. TNF, tumor necrosis factor  $\alpha$ ; TPEN, N,N,N',N'-tetrakis (2-pyridylmethyl)-ethylene diamine; Zn, zinc; TZD, thiazolidinedione; CF, ciprofibrate; FF, fenofibrate; concentrations in  $\mu$ mol/L. Values are means  $\pm$  SEM (n = 3).

\*Significantly different compared to control and treatment groups as labeled (#).

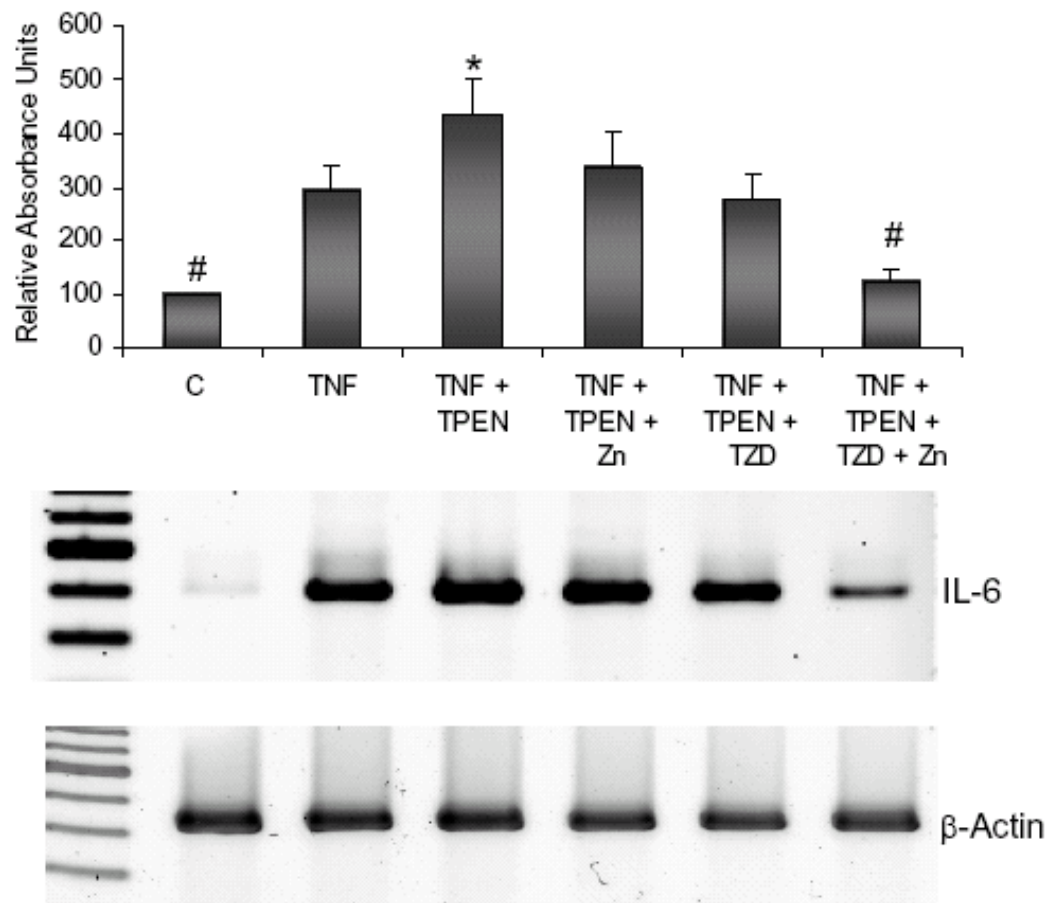


Figure 11, PPAR agonists require zinc to prevent IL-6 mRNA expression

Effects of TNF- $\alpha$ , zinc depletion/supplementation and PPAR agonists on IL-6 mRNA levels in endothelial cells. Endothelial cells were pretreated with PPAR agonists and zinc/zinc chelator TPEN (18 h) and exposed to TNF- $\alpha$  for 2 h.  $\beta$ -actin was used as a housekeeping gene. TNF, tumor necrosis factor  $\alpha$ ; TPEN, N,N,N',N'-tetrakis (2-pyridylmethyl)-ethylene diamine; Zn, zinc; TZD, thiazolidinedione; CF, ciprofibrate; FF, fenofibrate; concentrations in  $\mu\text{mol/L}$ . Values are means  $\pm$  SEM (n = 3).

\*Significantly different compared to control and treatment groups as labeled (#).

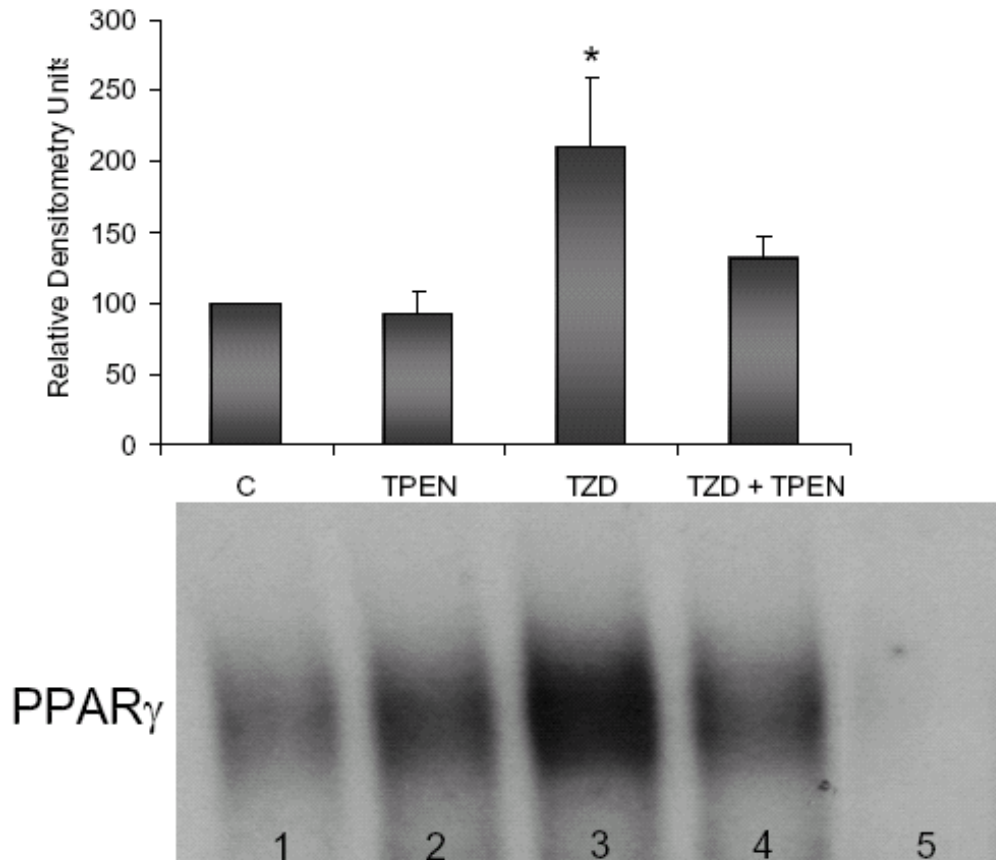


Figure 12, PPAR $\gamma$  agonists induce PPAR binding only in the presence of zinc

Effects of zinc depletion on PPAR $\gamma$  binding activity in endothelial cells. Primary endothelial cells were zinc deprived by chelation and treated with PPAR $\gamma$  agonist TZD. Lane 5 shows a super shift that confirms the depicted lane to be that of PPAR $\gamma$ . TNF, tumor necrosis factor  $\alpha$ ; TPEN, N,N,N',N'-tetrakis (2-pyridylmethyl)-ethylene diamine; Zn, zinc; TZD, thiazolidinedione; concentrations in  $\mu\text{mol/L}$ . Values are means  $\pm$  SEM (n = 3). \*Significantly different compared to all other treatment groups.

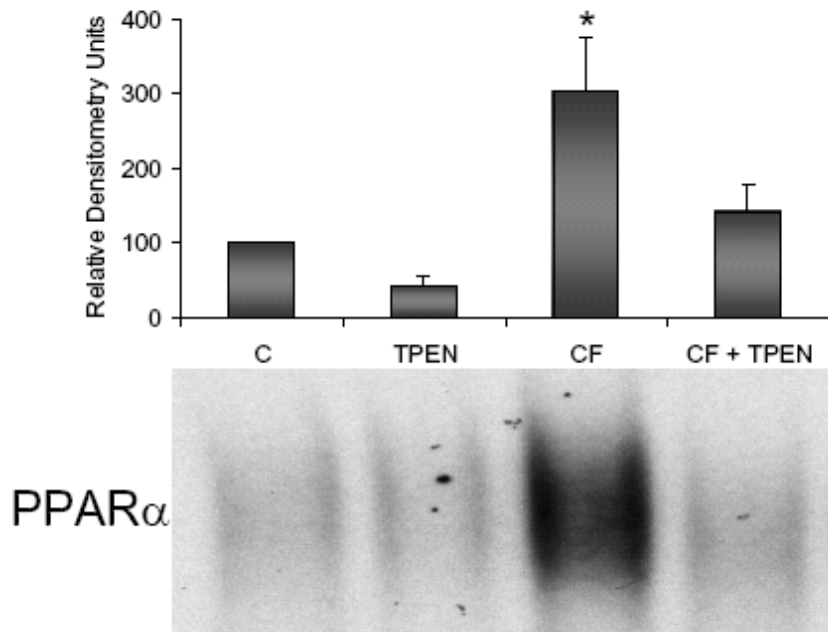


Figure 13, PPAR $\alpha$  agonists induce PPAR binding only in the presence of zinc

Effects of zinc depletion on PPAR $\alpha$  binding activity in endothelial cells. Primary endothelial cells were zinc deprived by chelation and treated with PPAR $\alpha$  agonist CF (B). TNF, tumor necrosis factor  $\alpha$ ; TPEN, N,N,N',N'-tetrakis (2-pyridylmethyl)-ethylene diamine; Zn, zinc; CF, ciprofibrate; concentrations in  $\mu\text{mol/L}$ . Values are means  $\pm$  SEM (n = 3). \*Significantly different compared to all other treatment groups.

## ***Discussion***

Zinc appears to act through different mechanisms. Zinc has been shown to be essential to the structure and function of a large number of macromolecules and for over 300 enzymatic reactions (Sandstead 1995). It has both catalytic and structural roles in enzymes, while in zinc finger motifs, it provides the scaffold that organizes protein sub-domains for the interaction with either DNA or other proteins (Sandstead 1995)

Zinc is a critical component of biomembranes and is essential for proper membrane structure and function (O'Dell 2000). In addition, zinc is required for cellular repair processes and could therefore play a critical role in protecting the vasculature from cell injury and loss of barrier function – both critical initial steps in the pathogenesis of atherosclerosis (Hennig et al. 2001). In the current study we show that adequate amounts of zinc are required for the anti-inflammatory properties of PPAR $\alpha$  and  $\gamma$  agonists. In zinc deficient cells, PPAR agonists failed to downregulate the TNF- $\alpha$  induced NF- $\kappa$ B and AP-1 transcription factor binding activity as well as the expression of the pro-inflammatory genes VCAM-1 and IL-6. TPEN alone did not induce any of the inflammatory markers measured (not all data shown) indicating that zinc deficiency primes cells for a higher susceptibility to inflammatory inducers, such as TNF- $\alpha$ , but does not induce inflammation by itself. The involvement of zinc in PPAR is a novel concept and the mechanism of the interaction is not known.

PPAR $\alpha$  and  $\gamma$  are nuclear receptors expressed in a wide variety of tissues, including vascular endothelial cells (Marx et al. 1998). PPARs regulate lipid and lipoprotein metabolism, glucose homeostasis as well as cell proliferation and differentiation. In addition, PPARs also modulate the inflammatory response. In addition to ligand binding, cofactor recruitment appears to play a key role in the transcriptional regulation by PPARs. In fact, it was reported recently that different subsets of cofactors are recruited to the PPAR transcription factor machinery dependent on the nature of the PPAR ligand (Torra et al. 2001). The difference in cofactors appears to contribute to a ligand specific response in gene transcription. Because zinc is known to be essential for protein structure, zinc might have importance in the assembly and stability of the PPAR-RXR-cofactor complexes.

In fact, the DNA binding domain of PPARs consists of two sets of zinc fingers (Gearing et al. 1994). The specificity and polarity of PPAR-DNA binding seems to be at least in part due to features in the zinc finger domains of PPAR (Hsu et al. 1998). The binding partner of PPAR, RXR, also has a DNA binding domain with two zinc fingers involved (Lee et al. 1993). As zinc is an essential constituent of the DNA binding domains of both PPAR and RXR, zinc deficiency could impair the function of this transcription factor complexes. In addition, as a critical component of the cells anti-oxidative defense system, zinc appears to be important for maintaining an environment that facilitates normal protein-protein interaction. It has been suggested, that zinc deficiency induces alterations in the intracellular redox state that can lead to the oxidation of thiol groups thus impairing protein function (Oteiza et al. 2001). In fact, zinc finger structures require two zinc-coordinated cysteine sulfhydryl groups, and oxidation of these can eliminate DNA-binding and transcriptional functions (Webster et al. 2001). This might be relevant for optimal functioning of the PPAR/RXR protein complex.

Although the effect of oxidative stress on PPAR is controversial, oxidative stress has been reported to down-regulate PPAR $\alpha$  mRNA in skeletal muscle cells (Cabrero et al. 2002). Zinc could change PPAR mRNA levels by preventing the generation of reactive oxidative species (ROS). Indeed, we reported recently that zinc deficiency decreased PPAR $\gamma$  mRNA and protein levels (Meerarani et al. 2003).

Overall, our data suggest that zinc is a potent anti-inflammatory substance, which can help protect the endothelium against inflammatory events mediated by TNF- $\alpha$ . The data further demonstrate the importance of zinc for the anti-inflammatory properties of PPAR $\alpha$  and  $\gamma$  agonists. In zinc deficient cells, several PPAR agonists tested failed to downregulate the activation of inflammatory transcription factors and the expression of inflammatory genes. Furthermore, the DNA binding activity of both PPAR $\alpha$  and  $\gamma$  to the PPRE was greatly impaired in cells deprived from zinc. Because dietary zinc intake of certain population groups is still below intake recommendations (Tapiero and Tew 2003), these data underline the importance of adequate zinc intake during the administration of PPAR-targeting drugs to treat chronic diseases such as hyperlipidemia or diabetes.

### **III. Zinc Deficiency Induces Atherogenic Events in LDL-R -/- Mice**

#### **Synopsis**

Low zinc concentration can be associated with an increased risk in cardiovascular diseases, however studies in appropriate mouse models have been lacking so far. We here report that proatherogenic events are induced by zinc deficiency in low density lipoprotein receptor knock-out (LDL-R<sup>-/-</sup>) mice on a diet rich in corn oil. Mice received either a zinc deficient (0 ppm zinc), a zinc adequate (30 ppm), or a zinc supplemented (100 ppm) diet for a 4 week period. Mice on the zinc deficient diet showed significantly increased levels of cholesterol and triglycerides in VLDL and HDL fractions. Zinc supplementation on the other hand decreased these lipid parameters when compared to control mice. We detected the highest levels of 8-isoprostanes and glutathione reductase mRNA in thoracic aorta in zinc deficient mice, indicating higher levels of oxidative stress in this treatment group. Furthermore, inflammatory markers, such as NF- $\kappa$ B and VCAM-1 were significantly increased in zinc deficient mice when compared to mice of the zinc adequate or supplemented groups. In addition, the zinc deficiency markedly reduced the DNA binding activity of PPAR in liver extracts. Interestingly, mRNA expression levels of PPAR $\gamma$  were significantly increased in thoracic aortae of zinc deficient mice, indicating an adaptation process to decreased PPAR signaling. These data provide *in vivo* evidence of zinc deficiency inducing proinflammatory events in an atherogenic mouse model. These data also suggest that adequate zinc may be a critical component in protective PPAR signaling during atherosclerosis.



## ***Introduction***

Cardiovascular diseases are a major health problem in industrialized countries and have a rising incidence in the non-industrialized part of the world (Hu et al. 2002). The pathogenesis is thought to begin with endothelial cell dysfunction that is manifested as an increase in the expression of specific cytokines and adhesion molecules. These cytokines and adhesion molecules are proposed to mediate the inflammatory aspects of atherosclerosis by regulating the vascular entry of leukocytes. Upon entry into vascular tissues, macrophages take up lipids thus forming the so-called foam cells (reviewed in Hennig et al. 2001). In more progressed forms of atherosclerosis, lipids also accumulate extracellularly, resulting in an atheroma that is at high risk of rupture. Such a plaque rupture can result in an acute coronary syndrome (Ross et al. 1999).

Causes for the development of atherosclerosis are usually of multiple nature. Lifestyle and nutrition can be closely linked to the onset and the pace of progression of atherosclerotic events. Current patterns of energy intake frequently show the portion of the daily calorie intake that is derived from fat to be well over 30% (Taubes et al. 2001). Such nutritional habits have been shown to result in hyperlipidemia and dyslipidemia. Epidemiological studies have shown that a high intake of linoleic acid (n-6) found in corn or canola oil seems to contribute to the overall risk for developing cardiovascular diseases (Kok et al. 1991).

Much less attention has been paid to micronutrients, and particularly to minerals. However, epidemiological studies suggest that in some population groups low serum levels of zinc are associated with coronary artery disease (Singh et al. 1997). Furthermore, zinc concentrations were significantly lower in atherosclerotic plaques of abdominal aortas of deceased patients with ischemic heart disease and acute myocardial infarction (Vlad et al. 1994). Mechanisms of the protective functions of zinc in the pathogenesis of atherosclerosis, including vascular cell dysfunction and the inflammatory response, are not clear.

We, and others have shown that zinc has antioxidant and anti-inflammatory properties (Connell et al. 1997, Meerarani et al. 2000, Hennig et al. 1999, Beattie and Kwun 2004). Zinc is a structural component of proteins that stabilizes thiol groups, thus

rendering proteins less prone to oxidation (Klotz et al. 2003). We and others have shown previously that oxidative stress can induce nuclear factor- $\kappa$ B (NF- $\kappa$ B), a pro-inflammatory transcription factor involved in mediating endothelial cell activation (reviewed by De Nigris et al. 2001). In the extracellular space free radicals can cause modification of macromolecules, particularly lipids (Stehbens et al. 1999). Certain oxidized lipids such as isoprostanes can be utilized as biomarkers indicating elevated systemic oxidative stress (Roberts et al. 2002).

In addition to its antioxidant properties, we recently reported that zinc appears to be essential for the protective properties of peroxisome proliferator activated receptors (PPARs)  $\alpha$  and  $\gamma$  in vascular endothelial cells (Reiterer et al. 2004b). PPARs are nuclear receptors that are involved in many metabolic pathways, including lipid and glucose metabolism (Torra et al. 2001). In addition, PPARs appear to have anti-inflammatory properties that can protect against endothelial cell activation (Zhou et al. 1999, Delerive et al. 1999). All of these features make PPARs attractive targets for pharmaceutical intervention of cardiovascular diseases. Indeed, clinical and experimental evidence suggests that PPAR activation decreases the incidence of cardiovascular diseases (Torra et al. 2001).

Zinc deficiency in atherosclerosis has never been studied in an animal model suitable for atherosclerosis research. The LDL-R  $-/-$  mice has been accepted as a good model because, like in humans, most cholesterol is carried in low density lipoproteins (LDL) (Daugherty et al. 2002). Here, we hypothesized that zinc supplementation would offer protection against atherosclerotic events by reducing oxidative stress and inflammatory markers, and by increasing PPAR activity.

## ***Materials and Methods***

### *Animals and diets*

The LDL-R-deficient mice used in this study were obtained from The Jackson Laboratory (Bar Harbor, ME; Stock Number: 002207). The study was performed in collaboration with Dr. Ruth MacDonald at the University of Missouri, Columbia, MI and

all procedures were in compliance with the IACUC guidelines of the University of Missouri. All mice were 5 weeks old when put on a moderate fat diet with varying amounts of zinc (Table 1). Mice were divided into 3 groups of 11 mice per treatment: 0 ppm zinc, 30 ppm zinc (control) and 100 ppm zinc. All diets were prepared at the University of Missouri, vacuum packed and stored at 4° C to prevent lipid oxidation. Body weights of all mice were determined before, after and throughout the study to ensure that diets would not cause significant weight changes. After completion of the study (4 weeks), animals were euthanized using intraperitoneal pentobarbital injections.

### *Zinc quantification*

Zinc analyses were performed in collaboration with Dr. Ruth MacDonald at the University of Missouri. Rear limbs of mice were removed at time of sacrifice. Skins were removed and limbs were stored at -20° C pending analysis. Livers were flash frozen after excision from the animal and stored at -20 until analysis. Femur and liver samples were placed in crucibles and dried in a 95° C oven for 48 h, ashed for 18 h at 500° C in a muffle furnace. Crucibles were allowed to cool in the furnace, 0.25 mL of ultrapure nitric acid was added and crucibles were heated gently to dryness. Crucibles were returned to the muffle furnace and reheated overnight at 500° C. Crucibles were transferred to a hot plate and 3 drops of concentrated HCL was added, followed by 1.0 mL of 1% HCl. Crucibles were heated gently to dissolve ash. Volume was adjusted to 10 mL with 1 % HCl and samples were read by flame atomic absorption at 213.9 nm (Browning et al. 1998).

### *Plasma cholesterol and lipoprotein profiles*

Blood was drawn from exposed hearts using heparinized syringes. Plasma was obtained by centrifugation of whole blood at 3750 rpm, 4°C, for 20 min. Plasma cholesterol content was determined enzymatically using a commercially available kit (Wako Chemicals USA, Inc., Richmond, VA).

The lipoprotein profile was determined by HPLC (Chemstation LC/MSD 1100, Agilent Technologies, Wilmington, DE). Chromatographic profile was monitored by Diode Array Detector at 280 nm and Mass Selective Detector (Agilent Technologies, Wilmington, DE). Electrospray and chemical ionization were used to detect triglycerides

and phospholipids, respectively. Proteins were detected by measuring absorbance at 280 nm.

#### *Immunohistochemistry for VCAM-1 expression in aortic roots*

Hearts were separated from thoracic aortae, cut horizontally, immersed in OCT embedding medium and frozen at -20 °C. 10 µm sections were cut on a cryostat (Microm HM505N, Carl Zeiss, Waldorf, Germany). Immunocytochemistry was performed as described previously (Daugherty et al. 2000). Briefly, endogenous peroxidase was inactivated using hydrogen peroxide (3%) in methanol. Samples were blocked in the serum of the secondary antibody host. Primary antibodies for VCAM-1 (PharMingen, San Diego, CA) were detected using biotinylated secondary antibodies and peroxidase ABC kits (Vectastain, Burlingame, CA). Aminoethylcarbozole was used as chromogen, and sections were counterstained with hematoxylin.

#### *Gene expression analysis*

Thoracic aortae were excised from mice and stored in RNAlater (Quiagen, Valencia, CA) until analysis. Total RNA was isolated from aortae using RNeasy (Quiagen, Valencia, CA) after removing surrounding adipose and connective tissues. cDNA was generated using the Reverse Transcription System (Promega, Madison, WI). Gene expression was determined by real-time PCR using the ABI Prism 7000 and Taq Man Universal PCR Master Mix (Applied Biosystems, Branchburg, NJ). Primers and probes were designed to VCAM-1 (gi: 31981429), PPAR $\gamma$  (gi: 6755137) and glutathione reductase (gi: 34785373) by the Primer Express 2.0 software (Applied Biosystems, Branchburg, NJ) and synthesized by IDT DNA (Coralville, IA). Probes were 3' labeled with a reporter (6-FAM<sup>TM</sup>) and 5' labeled with a quencher (TAMRA<sup>TM</sup>). Detection of 18S RNA utilized pre-developed assay reagents (Applied Biosystems, Branchburg, NJ).

#### *Transcription factor (NF- $\kappa$ B and PPAR $\gamma$ ) activation studies: electrophoretic mobility shift assay (EMSA)*

Left liver lobes were flash frozen and stored at -80 °C until analysis. Nuclear extracts containing active proteins were prepared according to the method of Dignam et al.

(Dignam et al. 1993). Nuclear extracts were incubated for 25 min with  $^{32}\text{P}$ -end-labeled oligonucleotide probes containing the enhancer DNA element for NF- $\kappa\text{B}$  (Promega, Madison, WI) or the PPAR response element (PPRE) (Santa Cruz, Santa Cruz, CA). Incubation at room temperature was performed in the presence of nonspecific competitor DNA. Following binding, the complexed and uncomplexed DNA in the mixture were resolved by electrophoresis in a 6.5% (w/v) non-denaturing polyacrylamide gel and visualized by autoradiography. Control reactions using supershift assay were performed to demonstrate the specificity of the shifted DNA-protein complexes for NF- $\kappa\text{B}$  and PPAR $\gamma$  respectively. All antibodies used were obtained from Santa Cruz (Santa Cruz, CA).

#### *Isoprostane quantitation*

Equal amounts of plasma were pooled from all animals within one treatment group for a final volume of 1 mL. Samples were shipped on dry ice to Dr. Jason Morrow, Vanderbilt University, Nashville, TN. 8-iso-PGF<sub>2</sub> was measured by stable isotope dilution gas chromatography negative ion chemical ionization mass spectrometry as described previously (Morrow et al. 1999).

#### *Quantitations and statistical analyses*

Numeric data were analyzed using SYSTAT 7.0 (SPSS, Inc., Chicago, IL). Comparisons between treatments were made by one-way ANOVA with post-hoc comparisons of the means made by Tukey least significance difference procedure. A statistical probability of  $P < 0.05$  was considered significant. Photomicrographs of VCAM-1 staining in aortic roots were analyzed using a double blind design. Uncropped pictures of same magnification were assigned random labels. Staining intensity of photomicrographs were then evaluated by 5 volunteers by ranking the specimen according to staining intensity.

## **Results**

### *The effect of dietary zinc on body weight and tissue zinc levels*

Dietary zinc deficiency significantly decreased total body weights throughout the 4 week period of the study (Figures 14A and 14B). No changes in weight gain were observed between control and zinc supplemented animals. Femur zinc content was significantly changed in accordance to the dietary zinc animals received. Zinc levels were significantly lower in zinc deficient mice and significantly higher in zinc supplemented mice when compared to control (Figure 15A). However, no change in zinc content of liver tissues could be observed (Figure 15B).

### *Zinc decreased serum total cholesterol and decreased triglycerides, cholesterol and phospholipids in lipoproteins*

Total cholesterol, as determined enzymatically, was decreased by zinc in a dose dependent manner with a significant difference between animals on the zinc deficient diet and animals on the zinc supplemented diet (Figure 16). Free cholesterol in lipoprotein fractions exhibited a similar pattern. While no change could be observed in the LDL fraction, free cholesterol was significantly reduced by zinc supplementation in the VLDL and HDL fractions when compared to the zinc deficient animals (Figure 17). Cholesterol esters were significantly higher in VLDLs and LDLs of zinc deficient mice compared to both, zinc adequate and zinc supplemented animals (Figure 18). Triglycerides were significantly different in VLDLs between all groups with the lowest levels in zinc supplemented animals. Triglycerides in the LDL fraction showed the same trend, but significance was only found in the zinc supplemented animals compared to the zinc deficient and zinc adequate mice (Figure 19). Phospholipids did not differ between treatment groups in the LDL fraction but were significantly different in the VLDL and HDL fractions of zinc supplemented and zinc deficient mice, with the highest levels in zinc deficient mice (Figure 20).

#### *Zinc deficiency increased oxidative stress*

Mice deficient in zinc (0 ppm) exhibited a significantly higher mRNA expression of the antioxidant enzyme glutathione reductase as determined by RT-PCR of RNA extracted from thoracic aortae (Figure 21). There was no difference in the expression of this gene between the zinc sufficient (30 ppm) and zinc supplemented (100 ppm) groups. At the same time, 8-iso-isoprostanes F<sub>2</sub> (PGF<sub>2</sub>) the most representative F<sub>2</sub>-isoprostanes, were elevated in the plasma of mice fed the zinc deficient diet (0 ppm) when compared with the zinc sufficient (30 ppm) and zinc supplemented (100 ppm) groups (Figure 22). F<sub>2</sub>-isoprostane data shown represent pooled plasma samples from 11 animals per treatment group.

#### *Zinc dependent changes in liver transcription factor activities*

Zinc deficiency significantly increased the DNA binding activity of the proinflammatory transcription factor NF- $\kappa$ B (Figure 23) in liver tissues. No difference in DNA binding activity of this transcription factor was observed when comparing zinc adequate (30 ppm) with zinc supplemented (100 ppm) animals. On the other hand, the anti-inflammatory transcription factor PPAR $\gamma$  was most activated in livers of control animals (30 ppm) with the lowest DNA binding levels being in zinc deficient animals (Figure 24).

#### *Zinc deficiency induced VCAM-1 mRNA and protein*

VCAM-1 mRNA expression was significantly elevated in thoracic aortae of mice fed a zinc deficient diet (0 ppm) compared to mice receiving a zinc adequate (30 ppm) or zinc supplemented (100 ppm) diet (Figure 25). VCAM-1 expression was confirmed by immunohistochemistry of aortic roots (Figure 26). Tissues of zinc deficient mice showed a significantly higher amount of immunostaining for VCAM-1 than tissues from zinc adequate or zinc supplemented mice.

#### *Zinc dependent changes in PPAR $\gamma$ mRNA expression*

PPAR $\gamma$  mRNA was significantly increased in thoracic aortae of animals fed a zinc deficient diet when compared with tissues from animals on a zinc adequate diet (Figure

27). PPAR $\gamma$  mRNA expression values from animals in the zinc supplemented group were close to those of the control group but were not significantly different compared to any of the other groups.



Ingredient (g/kg)	Zn 0	Zn 30	Zn 100
<u>Protein</u>			
Egg white	200	200	200
DL-Methione	3	3	3
<u>Carbohydrate</u>			
Cornstarch	397.5	397.5	397.5
Sucrose	100	100	100
Cellulose	35	35	35
Dyetrose	131	131	131
<u>Fat</u>			
Corn Oil	50	50	50
Safflower Oil	20	20	20
Choline Bitrate	2.5	2.5	2.5
<u>Minerals</u>			
Mineralmix*	50	50	50
Zinc	0	0.03	0.1
Vit Mix 93	10	10	10
Biotin	1	1	1

Table 1, Experimental diets

All components of the experimental diets are listed. Values refer to g ingredient per kg diet. \* Mineralmix used did not contain zinc. Zn 0, treatment group receiving 0 ppm dietary zinc, Zn 30, treatment group receiving 30 ppm dietary zinc, Zn 100, treatment group receiving 100 ppm dietary zinc.

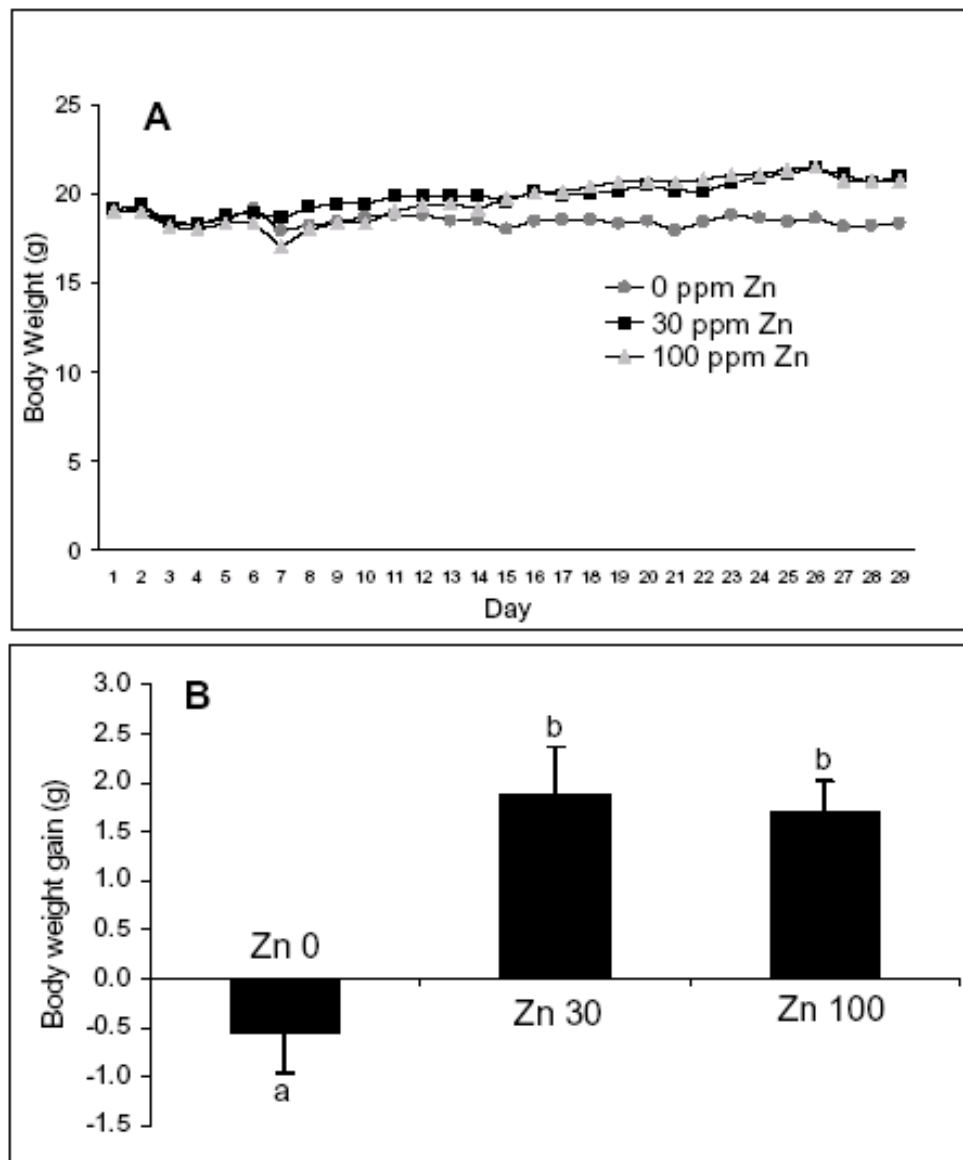


Figure 14, Mouse growth curve and body weight changes throughout the study

Body weights were obtained throughout the 4 week study period. Figure 14A shows the growth curve and Figure 14B depicts total weight changes from study start to study end. Values represent means  $\pm$  SEM. Zn 0, animals receiving diets containing 0 ppm zinc; Zn 30, animals receiving diets containing 30 ppm zinc; Zn 100, animals receiving diets containing 100 ppm zinc. Significant changes between treatment groups are labeled with (a) and (b) respectively.

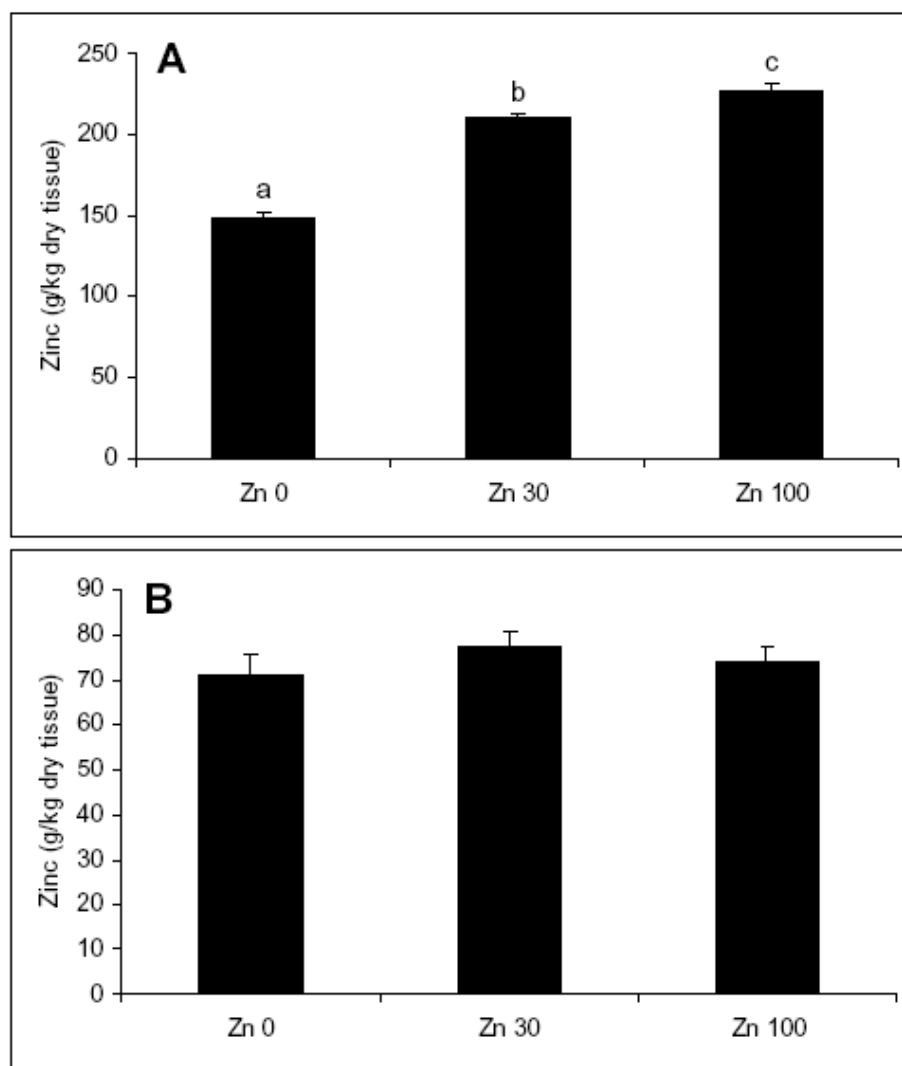


Figure 15, Zinc concentrations in femurs and livers

Zinc was measured from ashed tissue samples by atomic absorption methodology. Panel A shows femur zinc concentration whereas panel B shows liver zinc concentrations. Values represent means  $\pm$  SEM. Zn 0, animals receiving diets containing 0 ppm zinc; Zn 30, animals receiving diets containing 30 ppm zinc; Zn 100, animals receiving diets containing 100 ppm zinc. Significant changes between treatment groups are labeled with (a), (b) and (c) respectively.

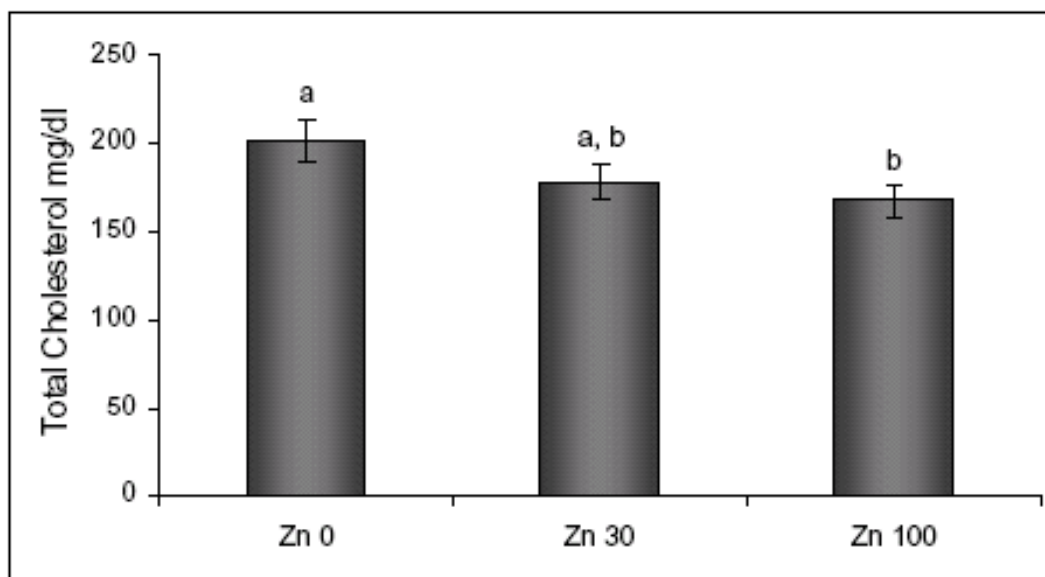


Figure 16, Effect of zinc on total serum cholesterol

Serum total cholesterol levels. Cholesterol levels were determined enzymatically.

Values represent means  $\pm$  SEM. Zn 0, animals receiving diets containing 0 ppm zinc; Zn 30, animals receiving diets containing 30 ppm zinc; Zn 100, animals receiving diets containing 100 ppm zinc. Significant changes between treatment groups are labeled with (a) and (b) respectively.

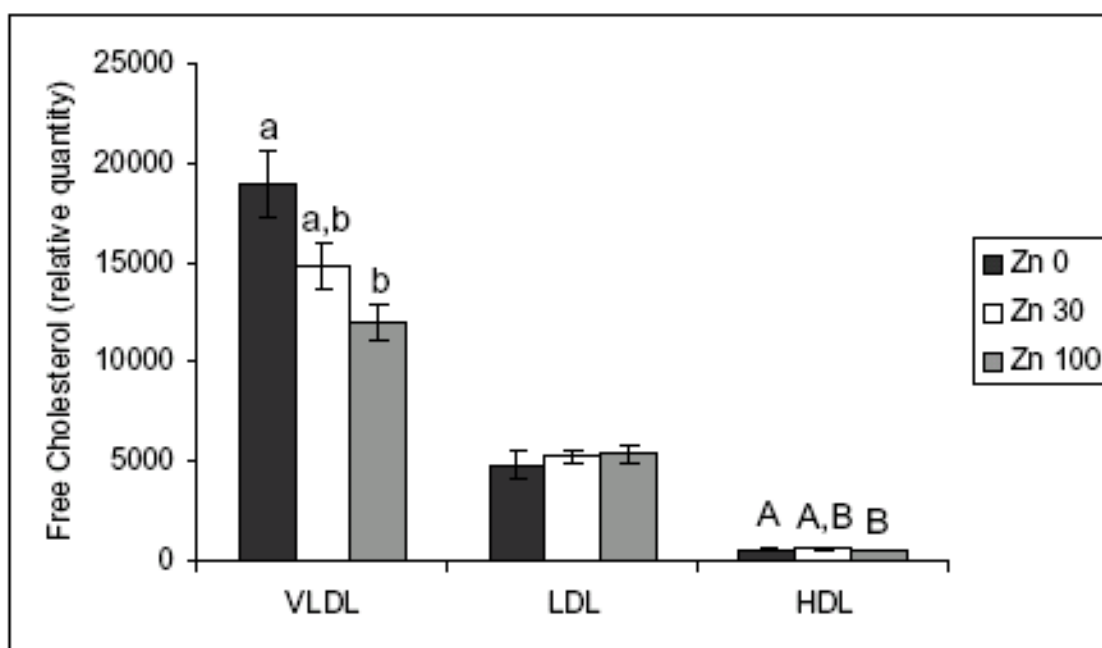


Figure 17, Zinc decreases free cholesterol in VLDL and HDL fractions

Free cholesterol in lipoprotein fraction. Lipoproteins were separated by size exclusion chromatography. Detection employed electron spray ionization and mass spectrometry. Values represent means  $\pm$  SEM. Zn 0, animals receiving diets containing 0 ppm zinc; Zn 30, animals receiving diets containing 30 ppm zinc; Zn 100, animals receiving diets containing 100 ppm zinc. Statistical analysis was performed between dietary groups within the individual lipoprotein fractions. Significant changes are indicated by small and capitalized letters respectively.

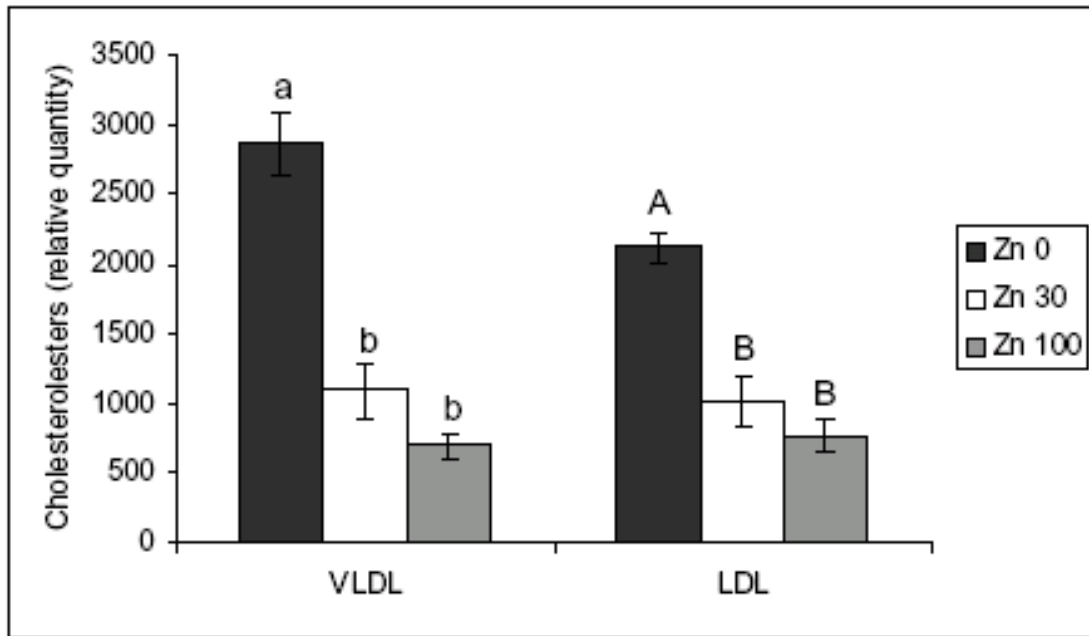


Figure 18, Zinc decreases cholesterol esters in VLDL and LDL fractions

Cholesterol esters in lipoprotein fraction. Lipoproteins were separated by size exclusion chromatography. Detection employed electron spray ionization and mass spectrometry. Values represent means  $\pm$  SEM. Zn 0, animals receiving diets containing 0 ppm zinc; Zn 30, animals receiving diets containing 30 ppm zinc; Zn 100, animals receiving diets containing 100 ppm zinc. Statistical analysis was performed between dietary groups within the individual lipoprotein fractions. Significant changes are indicated by small and capitalized letters respectively.

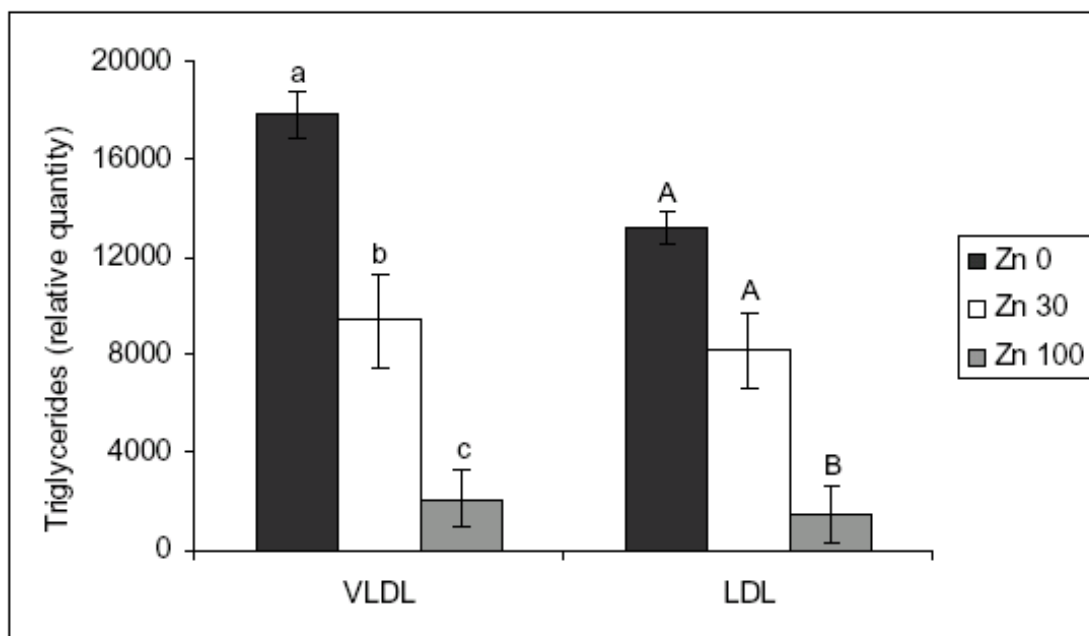


Figure 19, Zinc decreases triglycerides in VLDL and LDL fractions

Triglycerides in lipoprotein fraction. Lipoproteins were separated by size exclusion chromatography. Detection employed electron spray ionization and mass spectrometry. Values represent means  $\pm$  SEM. Zn 0, animals receiving diets containing 0 ppm zinc; Zn 30, animals receiving diets containing 30 ppm zinc; Zn 100, animals receiving diets containing 100 ppm zinc. Statistical analysis was performed between dietary groups within the individual lipoprotein fractions. Significant changes are indicated by small and capitalized letters respectively.

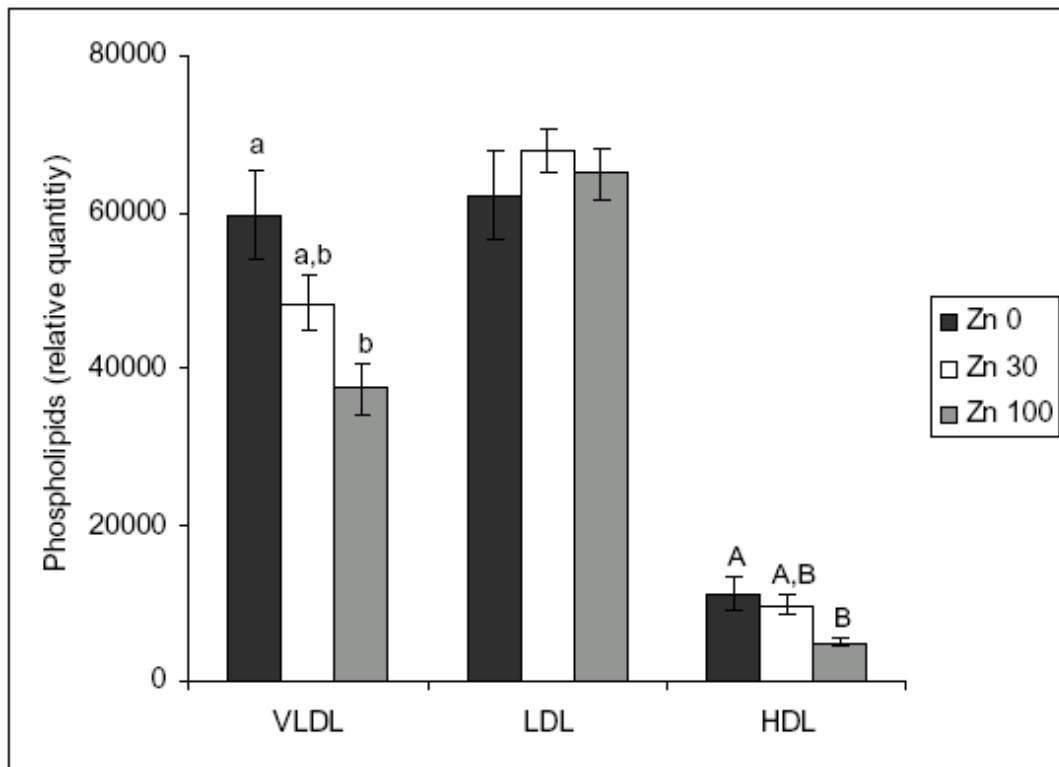


Figure 20, Zinc decreases phospholipids in VLDL and HDL fractions

Phospholipids in lipoprotein fraction. Lipoproteins were separated by size exclusion chromatography. Detection employed chemical ionization and mass spectrometry. Values represent means  $\pm$  SEM. Zn 0, animals receiving diets containing 0 ppm zinc; Zn 30, animals receiving diets containing 30 ppm zinc; Zn 100, animals receiving diets containing 100 ppm zinc. Statistical analysis was performed between dietary groups within the individual lipoprotein fractions. Significant changes are indicated by small and capitalized letters respectively.



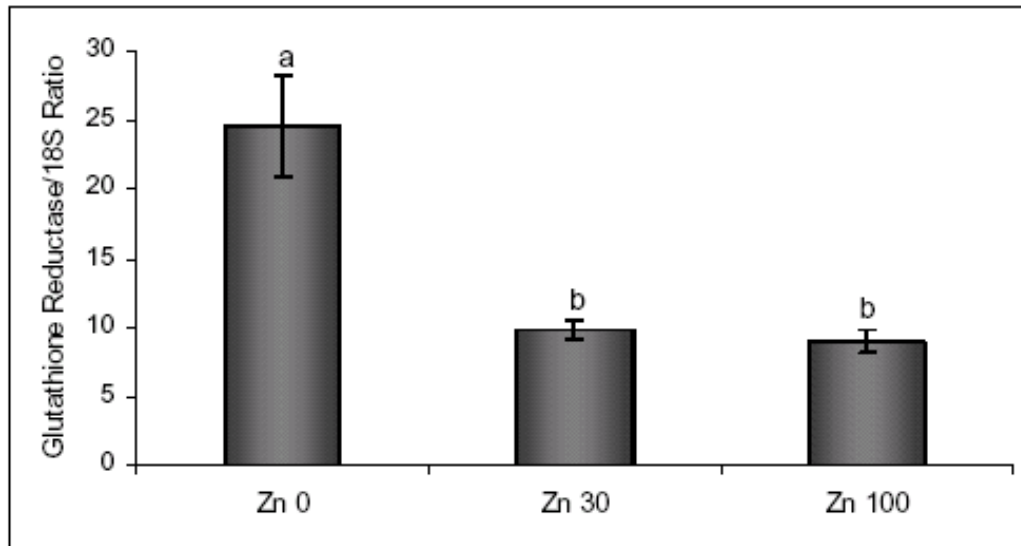


Figure 21, Glutathione reductase mRNA is upregulated during zinc deficiency

mRNA expression of glutathione reductase in thoracic aortae. Expression levels were determined by real-time PCR. Values shown represent mean expression data  $\pm$  SEM that had been normalized to expression levels of 18S RNA. Zn 0, animals receiving diets containing 0 ppm zinc; Zn 30, animals receiving diets containing 30 ppm zinc; Zn 100, animals receiving diets containing 100 ppm zinc. Dietary group labeled (a) was significantly different from other dietary groups (b).

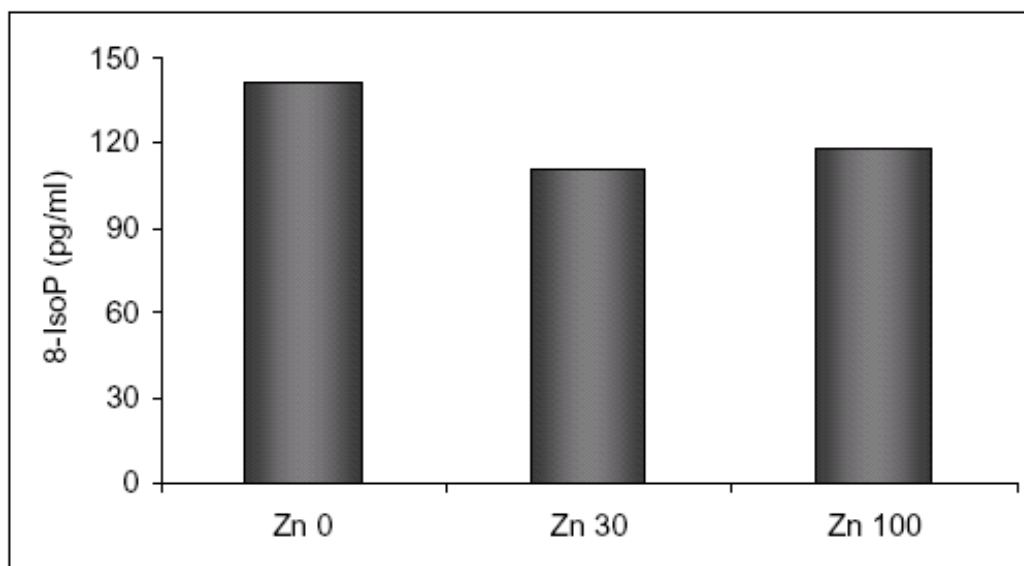


Figure 22, Increased formation of isoprostanes during zinc deficiency

Plasma isoprostane concentration. 8-iso-PGF<sub>2</sub> was quantified by gas chromatography. Data represent pooled samples from 11 animals for each of the respective treatment groups. Zn 0, animals receiving diets containing 0 ppm zinc; Zn 30, animals receiving diets containing 30 ppm zinc; Zn 100, animals receiving diets containing 100 ppm zinc.

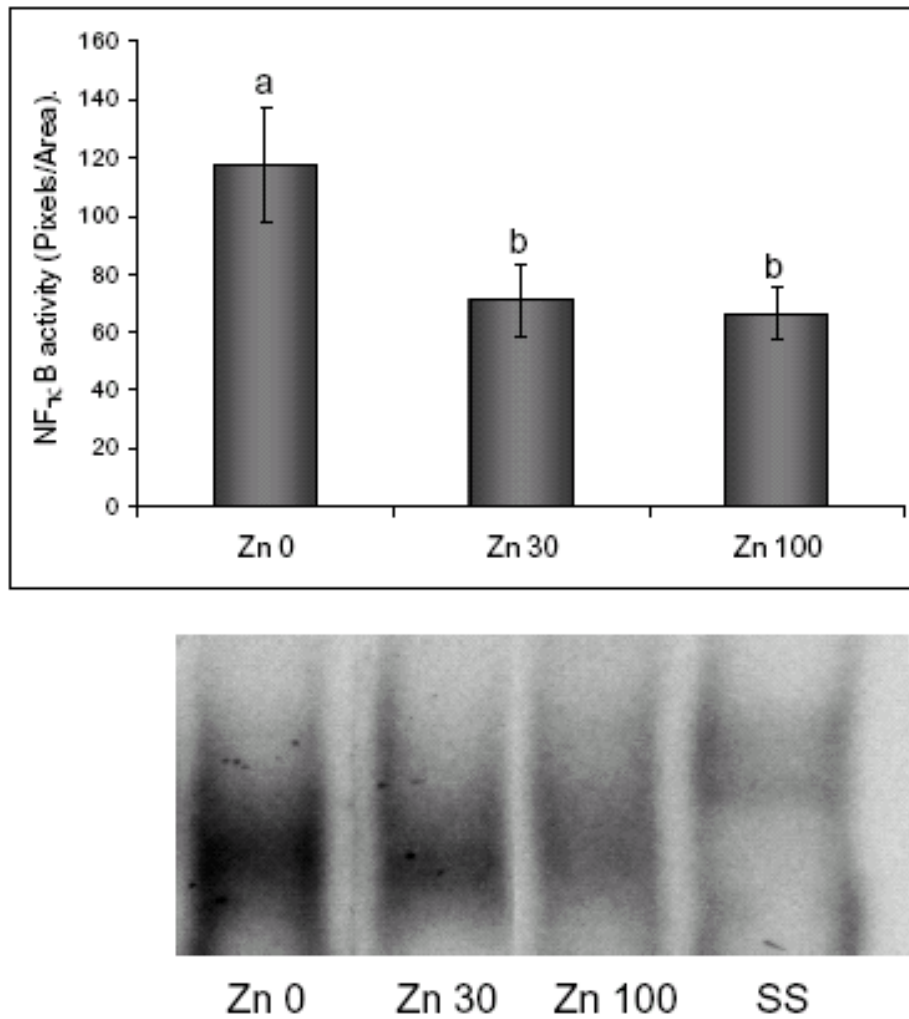


Figure 23, NF-κB DNA binding is increased in zinc deficient animals

DNA binding activity of NF-κB in liver extracts. Transcription factor activation was determined by gel shift assay (A) and bands visualized by autoradiography. Shown in 6B are mean densitometry units  $\pm$  SEM. Zn 0, animals receiving diets containing 0 ppm zinc; Zn 30, animals receiving diets containing 30 ppm zinc; Zn 100, animals receiving diets containing 100 ppm zinc; SS, supershift using antibodies specific for NF-κB p65. Dietary group labeled (a) was significantly different from other dietary groups (b).

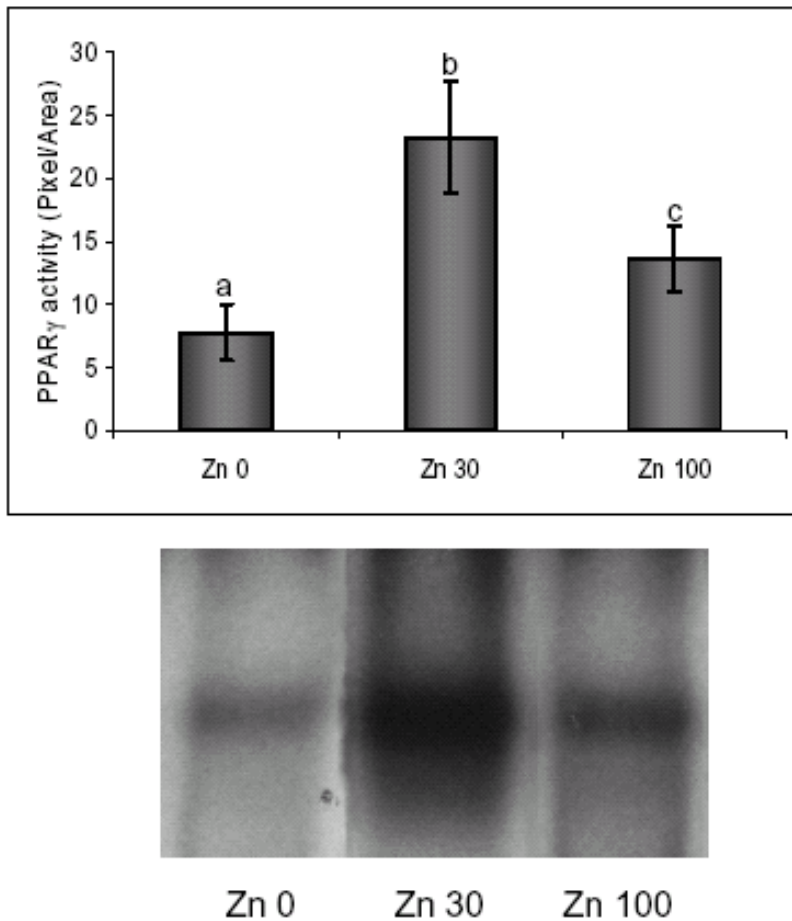


Figure 24, PPAR DNA binding is decreased in zinc deficient animals

DNA binding activity of PPARs to the PPRE in liver extracts. Transcription factor activation was measured by gel shift assay (A). Bands were quantified using densitometry (B). Values represent mean densitometry units  $\pm$  SEM. Zn 0, animals receiving diets containing 0 ppm zinc; Zn 30, animals receiving diets containing 30 ppm zinc; Zn 100, animals receiving diets containing 100 ppm zinc; SS, supershift with antibodies specific for PPAR $\gamma$  using the same protein sample as shown in Zn 30. All dietary treatment groups were significantly different from one another (labeled a, b, c).

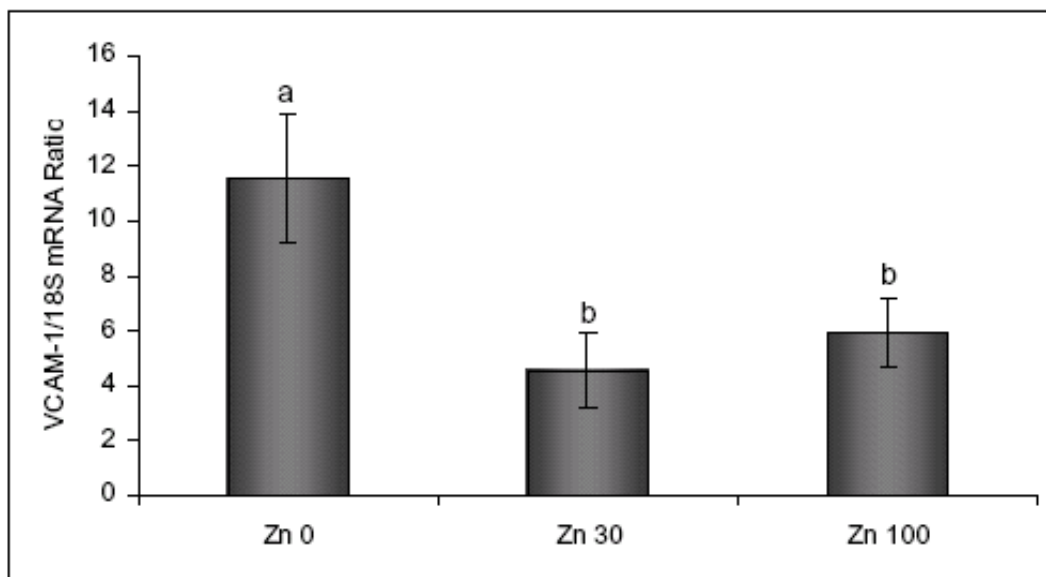


Figure 25, VCAM-1 mRNA is increased in thoracic aortae of zinc deficient mice

VCAM-1 mRNA expression in thoracic aortae. Gene expression was determined by real-time PCR. Values show mean  $\pm$  SEM of respective treatment groups normalized to expression levels of 18S RNA. Zn 0, animals receiving diets containing 0 ppm zinc; Zn 30, animals receiving diets containing 30 ppm zinc; Zn 100, animals receiving diets containing 100 ppm zinc. Dietary group labeled (a) was significantly different from other dietary groups (b).

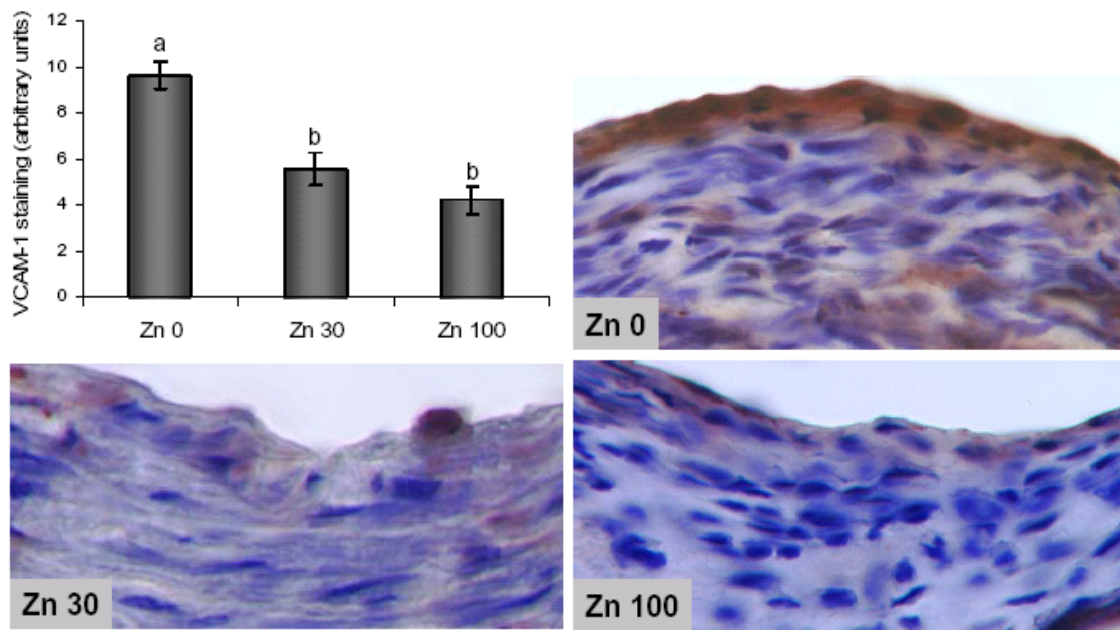


Figure 26, VCAM-1 protein is increased in thoracic aortae of zinc deficient mice

Protein expression of VCAM-1 in mouse aortic roots. VCAM-1 expression was detected by immunohistochemistry using primary antibodies against VCAM-1. Pictures are representative examples for the respective treatment group. Nuclei can be seen in blue and VCAM-1 staining on the endothelium in red. Graph shows amount of VCAM-1 expression as determined by ranking of slides according to the staining intensity (mean  $\pm$  SEM). Zn 0, animals receiving diets containing 0 ppm zinc; Zn 30, animals receiving diets containing 30 ppm zinc; Zn 100, animals receiving diets containing 100 ppm zinc; Dietary group labeled (a) was significantly different from other dietary groups (b).

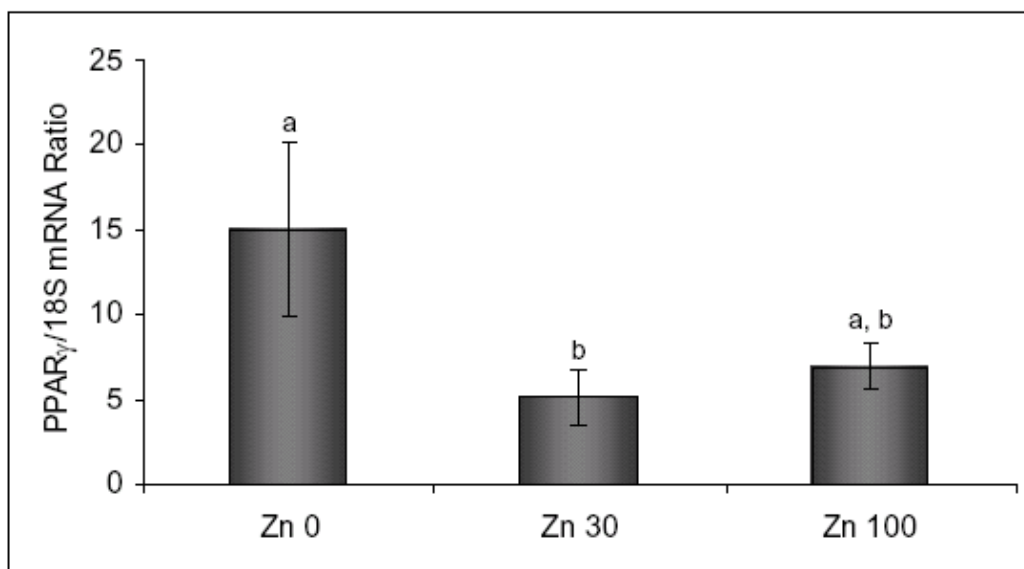


Figure 27, PPAR $\gamma$  mRNA is increased in zinc deficient mice

PPAR $\gamma$  mRNA expression in thoracic aortae. Gene expression was quantified by real-time PCR. Shown are mean expression values  $\pm$  SEM normalized to expression values of 18S RNA. Zn 0, animals receiving diets containing 0 ppm zinc; Zn 30, animals receiving diets containing 30 ppm zinc; Zn 100, animals receiving diets containing 100 ppm zinc. Dietary group labeled (a) was significantly different from dietary group labeled (b).

## ***Discussion***

Although zinc deficiency is more common in third world countries, dietary zinc intake is still below recommendations of certain population groups in industrialized countries (Tapiero et al. 2003). Zinc deficiency has been associated with growth retardation, impaired immune functions, delayed wound healing, and skin and taste abnormalities (Prasad et al. 1985). The role of zinc deficiency in atherosclerosis is not well defined, however, epidemiological studies suggest that in some population groups low serum levels of zinc are associated with coronary artery disease (Singh et al. 1997). By demonstrating that zinc deficiency induces atherosclerotic events, the results of the current study clearly add evidence to the importance of zinc in preventing atherosclerosis in LDL-R<sup>-/-</sup> mice on a medium-high fat diet.

Here we demonstrate the effect of dietary zinc content on femur and liver tissue zinc levels. Femur zinc was significantly modified by zinc deficiency and supplementation, respectively. On the other hand, we could not detect changes in the liver zinc levels. The liver is a more labile zinc pool when compared to the bone (King 1990). Our data indicate that during an extended period of zinc deficiency, zinc is mobilized from the bone storage to replenish zinc levels of soft tissues. However, the total zinc content of a given tissue does not reveal any redistribution of cellular zinc that might cause biological responses to zinc deficiency. Indeed, we found significant changes in liver transcription factor binding activities that were dependent on the dietary zinc intake.

The effect of zinc on plasma lipid levels has been controversial. Other investigators studying different model systems found increased levels of cholesterol, phospholipids, VLDL and IDL during zinc deficiency (Faure et al. 1991). On the other hand, it has been reported that zinc deficiency decreased plasma and VLDL triglyceride levels in rats (Koo and Williams 1981). However, this effect was mostly attributed to the lower food intake resulting from zinc deficiency. The effect of zinc deficiency has never been studied in a mouse model appropriate for studying atherosclerosis. In this study we utilized LDL-R<sup>-/-</sup> mice that have a similar lipoprotein profile compared to humans (Daugherty et al. 2002). Here, we observe a striking difference in cholesterol,



triglycerides, and phospholipids in lipoprotein fractions. The lipid lowering effect of zinc has also been shown in human subjects (Black et al. 1988). The mechanism for the effect of zinc on lipoproteins is not clear. It has been suggested that zinc is required for enzymes involved in lipid synthesis and lipoprotein excretion (Cunnane 1988). For example, Porsch-Ozcurumez et al. (Porsch-Ozcurumez et al. 2001) reported zinc finger protein 202 (ZNF202) to inhibit the expression of ATP binding cassette transporter A1 and G1, both responsible for cholesterol and phospholipids efflux.

Zinc has been shown to be essential to the structure and function of a large number of proteins (Barnadier et al. 1999), including the antioxidant enzyme SOD. Decreased levels of Cu, Zn-SOD activity have been reported to result from dietary zinc deficiency (Virgili et al. 1999). In fact, the increased mRNA expression levels of glutathione reductase we observed in this current study, could indicate an adaptation to a decreased activity of SOD. The upregulated expression of glutathione reductase in thoracic aortae of zinc deficient mice could also be a result from increased oxidative stress with resulting upregulation of genes involved in the defense against free radicals. The higher plasma isoprostane levels in mice on a zinc deficient diet also indicate an increase in oxidative stress due to zinc deficiency. Isoprostanes are prostaglandin-like compounds that are formed from the peroxidation of arachidonic acid and can be used as reliable and quantifiable markers of oxidative stress (Morrow et al. 1999).

Oxidative stress can induce inflammation and contribute to the progression of atherosclerosis. We and others have reported before that inflammatory pathways, such as the NF- $\kappa$ B pathway are sensitive to oxidative stress. We have shown in endothelial cell cultures that zinc deficiency can induce NF- $\kappa$ B DNA binding activity (Hennig et al. 2002). Here we report *in vivo* evidence for the zinc deficiency-mediated induction of NF- $\kappa$ B activity in liver extracts. NF- $\kappa$ B has a key role in the transcription of inflammatory genes that induce endothelial cell dysfunction and thus mediate monocyte recruitment. VCAM-1 is a very important example of a NF- $\kappa$ B target gene that is involved in leukocyte attachment to the endothelial cells and diapedesis through the endothelium (Cook-Mills et al. 2002). The fact that VCAM-1 mRNA and protein expression were upregulated in aortic tissues of mice fed a zinc deficient diet indicates that NF- $\kappa$ B was most likely activated not only in livers but also in the vasculature. In

deed, we have previously observed a marked induction of NF- $\kappa$ B in cultured endothelial cells during zinc deficiency (Connel et al. 1997)

NF- $\kappa$ B activation and VCAM-1 expression have been reported to be inhibited by PPAR $\alpha$  and  $\gamma$  agonists (Zhou et al. 1999, Delerive et al. 1999). PPARs are nuclear receptors expressed in a wide variety of tissues, including vascular endothelial cells (Delerive et al. 1999). We have reported recently that zinc is required for the anti-inflammatory properties of PPAR $\alpha$  and  $\gamma$  agonists against TNF-induced endothelial cell activation (Reiterer et al. 2004b). In the current study we show that PPAR binding activity in liver extracts is dependent on the amount of zinc present. Zinc deficiency decreased PPAR DNA binding activity in liver extracts. We also have *in vitro* data showing that PPAR $\alpha$  and  $\gamma$  agonists were unable to induce PPAR binding activity in zinc deficient endothelial cells (Reiterer et al. 2004b).

It is unclear why zinc supplementation decreased liver PPAR binding activity compared to the control diet. Because the liver is the main organ of lipoprotein metabolism and because PPARs are known to be inducible by lipids (Torra et al. 2001), it is possible that the PPAR activity in the liver is closely related to lipid metabolism. Animals receiving the zinc supplemented diet had the lowest serum cholesterol and triglyceride concentrations. Thus lower PPAR DNA binding activities in animals supplemented with zinc compared to mice fed the zinc adequate diet might be due to less lipid induced PPAR activation. On the other hand, PPAR $\gamma$  expression in thoracic aortae was significantly increased in zinc deficient mice compared to mice on a zinc sufficient or zinc supplemented diet. A higher mRNA expression could indicate an attempt to compensate for proteins with decreased function. Our previous endothelial cell culture studies and our current results in liver and aortic tissues confirm that PPAR signaling appears to be impaired during zinc deficiency.

We are providing important *in vivo* evidence that zinc deficiency induces atherosclerotic events in a mouse model that is prone to develop atherosclerosis when kept on a high fat diet. The data are particularly valuable as this mouse model with LDL receptor deficiency shows a lipoprotein profile comparable to that of humans. Thus the data appear to be of relevance for human health. Because dietary zinc intake of certain population groups is still below intake recommendations (Tapiero et al. 2003), these data

underline the importance of zinc for the prevention of cardiovascular diseases such as atherosclerosis. In addition, adequate zinc status appears to be necessary during the administration of PPAR-targeting drugs to treat chronic diseases such as hyperlipidemia or diabetes.

## Conclusion

Atherosclerosis can be modified by nutrition. In the current work we studied the role of certain nutrients in the development of and protection against endothelial cell dysfunction. This initial step in atherogenesis is the time point where nutrition can play an important part in modifying the pathology of cardiovascular diseases.

All nutrients studied in this dissertation can be linked to each other by playing a role in oxidative stress: linoleic acid as a polyunsaturated fatty acid prone to oxidation that could contribute to oxidative stress, quercetin and vitamin E as antioxidants that can directly quench free radicals, and zinc as a structural component of proteins that stabilizes thiol groups, thus rendering proteins less prone to oxidation (Klotz et al. 2003).

Oxidative stress has a dual role in atherosclerosis. Extracellular oxidative stress leads to the formation of oxidized macromolecules, particularly lipids that act on cell surface receptors to induce their uptake by macrophages and to induce intracellular signaling pathways in endothelial cells. Isoprostanes, as studied in chapter three of this dissertation, are also known to act on extracellular receptors where they elicit their biological effects, including vasoconstriction or fibroblast proliferation (Mezzetti et al. 2000). On the other hand, intracellular free radicals appear to play an active role in inducing so called oxidative stress sensitive pathways. Although the mechanism is not clear, oxidized macromolecules could also mediate intracellular oxidative stress to certain signaling events. As it is mentioned in chapter one of this dissertation, native versus oxidized fatty acids appear to have different potencies in activating PPAR $\gamma$ . In proteins, thiol groups in cysteine moieties have been suggested to be involved in mediating intracellular oxidative stress (Chen et al. 2003). For example, the transcription factor AP-1, that was also studied in this dissertation, is partly activated by apoptosis signal-related kinase-1 (ASK-1). ASK-1 forms an inactive thioredoxin complex that can be reversed by ROS in response to TNF- $\alpha$  thus creating the activated form of this molecule (Saitoh et. Al 1998).

However, the role of linoleic acid, zinc and quercetin in inflammation is not exclusively mediated by their pro- or antioxidant properties. Our laboratory found that linoleic acid can initiate signaling events at the level of cell membranes. These signaling

events include the activation of phosphatidylinositol 3-kinase (PI3K) and extracellular signal regulated kinase (ERK) that, following a cascade of protein phosphorylations, elicit their effects in activating NF- $\kappa$ B and the transcription of cytokines and adhesion molecules (unpublished data). Nutrients, such as quercetin could interfere with these signaling cascades at several levels as it is known to possess kinase inhibitory properties (Peet and Li 1999, Yoshizumi et al. 2002). The anti-inflammatory properties of zinc are not well understood. However, we have demonstrated before that zinc can downregulate inflammation in response to TNF- $\alpha$  (Hennig et al. 1999, Connell et al. 1997, Meerarani et al. 2000). Here we report that the anti-inflammatory properties of zinc might be partly mediated by PPAR $\alpha$  and  $\gamma$  (Reiterer et al. 2004b).

Also linking all three chapters of this dissertation are PPARs. PPARs are categorized within the nuclear receptors as orphan receptors, because their natural ligands are still insufficiently defined (Van Bisen et al. 2002). The roles of PPAR $\alpha$  and  $\gamma$  have been studied mostly by targeting them with pharmaceutical compounds. Thus the effects of PPARs on lipid and glucose metabolism as well as their role in inflammation has been well established. However, natural ligands appear to be proinflammatory molecules such as eicosanoids or oxidized lipids (Van Bisen et al. 2002). Thus, the innate role of PPARs in inflammation could be of a negative-feedback nature. Molecules activating proinflammatory pathways, such as the NF- $\kappa$ B and AP-1 pathways also induce PPARs that can counter regulate these inflammatory events. Thus, the advantage of synthetic ligands that only induce PPARs and not inflammatory pathways becomes apparent.

The role of zinc in PPAR signaling is not well defined. As mentioned in the discussions to chapters two and three, PPARs contain zinc fingers that mediate the DNA binding (Gearing et al. 1994). However, zinc complexed by proteins that require zinc as a structural component might not be susceptible to zinc deficiency. This is also demonstrated by the fact that NF- $\kappa$ B binding activity is not decreased during zinc deficiency, despite the fact that NF- $\kappa$ B contains zinc fingers in the DNA binding domain as well. In fact, cellular zinc can be divided into a fixed pool, where zinc is tightly bound within tertiary protein structures and into a more labile pool that is more responsive to zinc supplementation and depletion (Truong-Tran et al. 2000). Zinc deficiency might therefore be more relevant for cellular processes that require free zinc. It is possible, that

the assembly of PPARs with other cofactors and coactivators requires the recruitment of one or more free zinc atoms, which would make the transcriptional activity of PPARs highly susceptible to zinc deficiency. In addition, protein half life could determine the susceptibility of a protein to zinc deficiency. If zinc is required for proper folding of a newly synthesized zinc-dependent protein, then a high turn-over (or short half life) would render this protein vulnerable to zinc deficiency. In deed, PPARs appear to have a rather short half life due to degradation following transcriptional activity (Blanquart et al. 2003). Furthermore, we observed an increase of PPAR $\gamma$  mRNA in aortae of zinc deficient mice, which could indicate an attempt to compensate for the loss of function of PPAR protein during zinc deficiency. It is therefore assumed that the effect of zinc on PPAR is at the actual PPAR level, rather than at any upstream level, for example proteins involved in PPAR transcription and expression.

In conclusion, data presented in this dissertation are novel and of importance for population groups at risk for zinc deficiency and for patients receiving pharmacological compounds targeting PPAR $\alpha$  or  $\gamma$ . Results shown also emphasize the importance of nutrition in the prevention and the importance of an adequate nutritional status during the treatment of cardiovascular diseases.

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97. Zilversmit D. B. (1979) Atherogenesis: a postprandial phenomenon. *Circulation.*  
60:473-85.



# Curriculum Vitae

## Gudrun Reiterer

Date and Place of Birth: 04/03/1978, Leoben, Austria

## Education

1. B.S./M.S. University of Vienna. 08/1996 – 06/2000.  
Major: Nutritional Sciences  
Minor: Nutrition Economics  
Advisor: Ibrahim Elmadfa, Ph.D.

## Research and Professional Experience

1. Research Assistant in the Department of Agriculture, Food and Nutritional Science  
University of Alberta, Canada. 09/2000 – 07/2001.
2. Research Assistant in the Ludwig-Boltzmann Institute,  
East Vienna Social Medicine Center 10/1999 – 08/2000.

## Academic Honors

1. New Investigator Award.  
I was chosen by the award committee of the American College of Nutrition to receive this award for my research on the involvement of zinc in PPAR signaling. I accepted this award at the annual meeting of the American College of Nutrition in Long Beach, CA, Sept 30 – Oct 3, 2004.

2. Award for Best Toxicological Poster. 06/16/2004.

I was the winner of the poster competition at the 2004 PCB Workshop in Urbana-Champaign, IL.

3. American Heart Association Predoctoral Fellowship. 07/2002 – 06/2004.

I received this stipend from the American Heart Association for my research proposal on Zinc, PPAR and Endothelial Cell Activation. In addition, the Graduate School of the University of Kentucky awarded me with the Graduate School Incentive Award for receiving extramural funding.

4. Commonwealth Research Award. 04/2004.

The Graduate School of the University of Kentucky granted me the Commonwealth Research Award to finance my travel to the Experimental Biology meeting, Washington, DC in April 2004, where I presented three posters. In addition, the Graduate School of the University of Kentucky supported travel to several other professional meetings and conferences: Experimental Biology meeting, New Orleans in April 2002, and the annual meeting of the American College of Nutrition, San Antonio in August 2002.

5. Dissertation Enhancement Award. 02/2003.

I was granted this award by the Graduate School of the University of Kentucky to receive training in gene silencing with small interfering RNA. I attended a hands-on workshop in San Diego, CA.

6. IAESTE – stipend. 09/2000 – 07/2001.

I was elected by the IAESTE (International Association for the Exchange of Students for Technical Experience) – committee based on my qualifications required for the position at the University of Alberta in Edmonton, Canada.

7. Academic Award from the University of Vienna. 10/2000.

This scholarship was based on my grade point average and the fact that I could complete the required course work for the Master's Program in Nutritional Sciences in an unusual short period of time.

## **Publications**

1. **Reiterer G** et al. Zinc deficiency induces atherogenic events in LDL-R <sup>-/-</sup> mice on a moderate-high fat diet (in preparation).
2. Hennig B, **Reiterer G**, Toborek M, Meerarani M., Daugherty A, Smart E, Matveev S, Robertson LW. A diet high in corn oil modifies PCB-induced changes in plasma and tissue lipid profiles in LDL receptor deficient mice. EHP (in press).
3. **Reiterer G**, Toborek M., Hennig B. PPAR alpha and gamma require zinc for their anti-inflammatory properties in vascular endothelial cells. J Nutr. 2004, 134:1711-5.
4. **Reiterer G**, Toborek M, Hennig B. Quercetin protects against linoleic acid-induced porcine endothelial cell dysfunction. J Nutr. 2004, 134:771-5.
5. Meerarani P, **Reiterer G**, Toborek M, Hennig B. Zinc modulates PPARgamma signaling and activation of porcine endothelial cells. J Nutr. 2003, 133:3058-64.